Serotonin Deficiency in Phenylketonuria Embryopathy

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Abstract—The development and evolution of PKU can be prevented by prescribing an appropriate diet at an early age. A systematic neonatal screening has been set up in most countries. However, young women suffering from PKU give birth to very severely malformed children (PKU embryopathy: microcephaly, mental retardation, hypotrophy, cardiopathy) unless they again take up the specific diet, until the PHE level has lowered down to normal, before the beginning of gestation. The treatment has to be continued at least during the first months of gestation. This management is very unpleasant and sometimes not easily accepted. The mechanism of this embryopathy is still unknown. It is possible that (1) the excess of PHE or the presence of abnormal metabolites, or (2) serotonin deficiency (which is a feature of PKU) could be responsible for the maldevelopment of the embryo. Some authors consider that serontonin has a morphogenetic role in normal embryogenesis. Previously we described an experimental animal model using *in vitro* culture of rat embryos in human PKU sera. Mouse embryos have been subsequently used, since they show a greater sensitivity. Malformations, consisting essentially of neural tube defects, were present in almost 100% of the embryos cultured in serum from PKU patients. Using this animal model, we tested the hypothesis of serotonin deficiency. For this purpose, mouse embryos were cultured in human serum depleted of serotonin. Under these conditions, 100% of the embryos showed oculo-neural malformations characteristic of the experimental embryopathy. These results indicate the importance of serotonin deficiency in the occurrence of PKU embryopathy.

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive disease that is caused by a deficiency of a liver-specific enzyme, phenylalanine hydroxylase (PAH) which catalyses the transformation of phenylalanine (Phe) to tyrosine. To date, more than 150 mutations have been reported at the genomic DNA level that result either in the loss of enzyme activity or in more or less significant levels of residual PAH activity. In France, where the population is of many different origins, mass neonatal screening for PKU has led to the observation of a large spectrum of variant forms of the disease related to both genetic heterogeneity and compound heterozygosity at the PAH locus.

A deficiency in the PAH activity results in accumulation of Phe which, in severely affected patients, increases above 1200 μmol (20 mg/100 ml) and causes severe intellectual deterioration unless a dietary treatment is introduced early in life. In contrast, in subjects with PAH residual activity, the Phe plasma level is, on a normal diet, below 600 μmol (10 mg/ml); in these cases normal development usually occurs without treatment. From the less to the most severe enzymatic defect no clear-cut distinction can be established such that there is a continuum in the phenotypes observed.

When necessary, the low Phe diet has to be strictly maintained for at least 6 or 7 yr. Some reports support the necessity of maintaining dietary treatment throughout adolescence, but others found no evidence of deterioration when therapy was discontinued at 5–8 yr of age (Scriver *et al.*, 1989).

Nevertheless, females have to return to very strict dietary control before conception. In fact, girls that have been correctly treated for PKU are quite normal when they reach childbearing age, but, if they are not treated again, the babies they bear have permanent abnormalities. Therefore it appears that the maternal dysmetabolism is teratogenic. PKU embryopathy consists of mental deficiency, microcephaly, cardiopathies and hypotrophy (birth weight <2500 g). There is an obvious correlation between the rate of malformations and the level of maternal phenylalaninaemia (Table 1, from Lenke and Levy, 1980). This embryopathy can be prevented by recommencing the dietary treatment long enough before gestation so that the maternal Phe has lowered to a normal level at the time of conception. This can take a long time and the treatment has to be continued for the whole of the gestation period. However, the diet is very

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Abbreviations: GD = gestation day; HIAA = 5-hydroxyindol acetic acid; 5-HT = serotonin; HVA = homovanillic acid; NTD = neural tube defect; PAH = phenylalanine hydroxylase; Phe = phenylalanine; PKU = phenylketonuria.

Table 1. PKU embryopathy*

Complication	Frequency (%) of affected pregnancies in PKU patients with maternal phenylalanine levels (mg/100 ml) of:				Frequency in normal population (%)
	> 20	16–19	11–15	3–10	
Spontaneous abortions	24	30	0	8	15–20
Mental retardation	92	73	22	21	5.0
Microcephaly	73	68	35	24	4.8
Congenital heart disease	12	15	6	0	0.8
Birth weight < 2500 g	40	52	56	13	9.6

*Data from Lenke and Levy (1980), who reported an international survey of the outcome of untreated and treated pregnancies among PKU patients.

unpleasant and frequently the patients do not regularly keep to it. Moreover, it is well known that many young women do not strictly comply with contraceptive prescriptions.

It has been said that unless 100% effective prevention is obtained, the benefit of systematic screening could be lost within a few decades. The efficiency of prevention partly depends on the understanding of its mechanisms, and at present these are mostly unknown. Hypotheses are based on the consequences of the PAH deficiency. As a consequence of hyperphenylalaninaemia, secondary abnormalities appear. (1) Abnormal metabolites are formed, resulting from the initiation of an accessory metabolic pathway which is normally inactive. These abnormal metabolites are mostly found in the urine. (2) Another important consequence of hyperphenylalaninaemia is secondary inhibition of the hydroxylases of the two other aromatic amino acids, tyrosine (TH) and tryptophan (TPH). These three enzymes (PAH, TH and TPH) are very similar. Tyrosine hydroxylation leads to formation of L-DOPA and catecholamines. Tryptophan hydroxylations leads to serotonin (5-HT). Actually, hyposerotoninaemia, which is correlated with the Phe level, is frequently observed in PKU patients (Fig. 1).

Therefore there are two main hypotheses to explain PKU embryopathy:

(1) 'intoxication' by hyperphenylalaninaemia and/or its abnormal metabolites.
(2) a deficiency in serotonin and/or catechol amines.

Certain data, presented later in this paper, suggest that serotonin and/or catecholamines could be effective precocious morphogens. We chose to test the hypothesis of the teratogenic action of a serotonin deficiency.

MATERIALS AND METHODS

Experimental model

We used the technique of post-implantation whole embryo culture, according to New (1978). The medium was pure human serum to which glucose (1 g/litre) was added and was either from normal subjects (for control embryos) or from untreated PKU patients (for test embryos). Normal human serum was provided by the CNTS (National Center of Blood Transfusion). For each human PKU serum, the levels of phenylalaninaemia and serotoninaemia were determined.

Rats, provided by Iffa-Credo (Saint Germain-sur-l'Arbresle, France), were of the Wistar strain. The virgin females weighed about 190–200 g on arrival. They were caged in groups of five, fed UAR AO3 and maintained in a 12/12-hr artificial dark/light cycle (light, 17.00–05.00 hr). Mating took place on the Friday morning between 08.00 and 10.00 hr (one

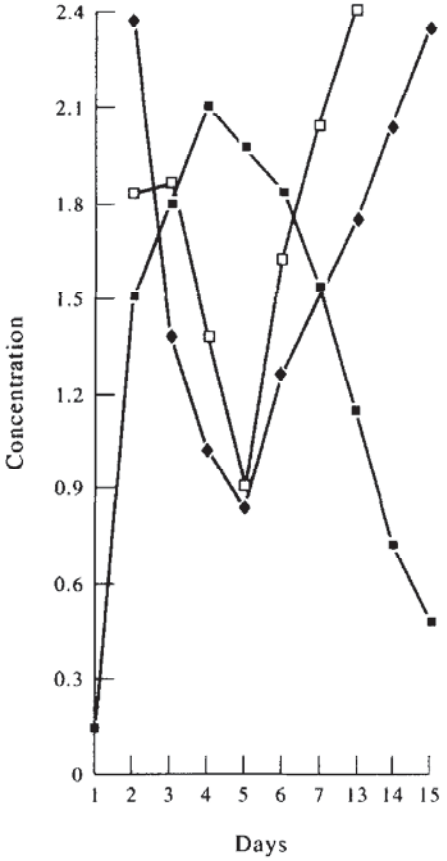

Fig. 1. Evolution of serotoninaemia according to the Phe level. On day 1, the patient is on dietary treatment. Phe is < 5 mg, the level of 5 HIAA and HVA (two urinary 5-HT metabolites) is normal. From day 1 to day 4 the patient is given a normal diet. The Phe level increases rapidly while the 5-HT level decreases. This phenomenon is reversible when the diet is reintroduced. ■, phenylalanine (mmol/litre); □, 5-HIAA (mmol/mol creatinine); ◆, HVA (mmol/mol creatinine).

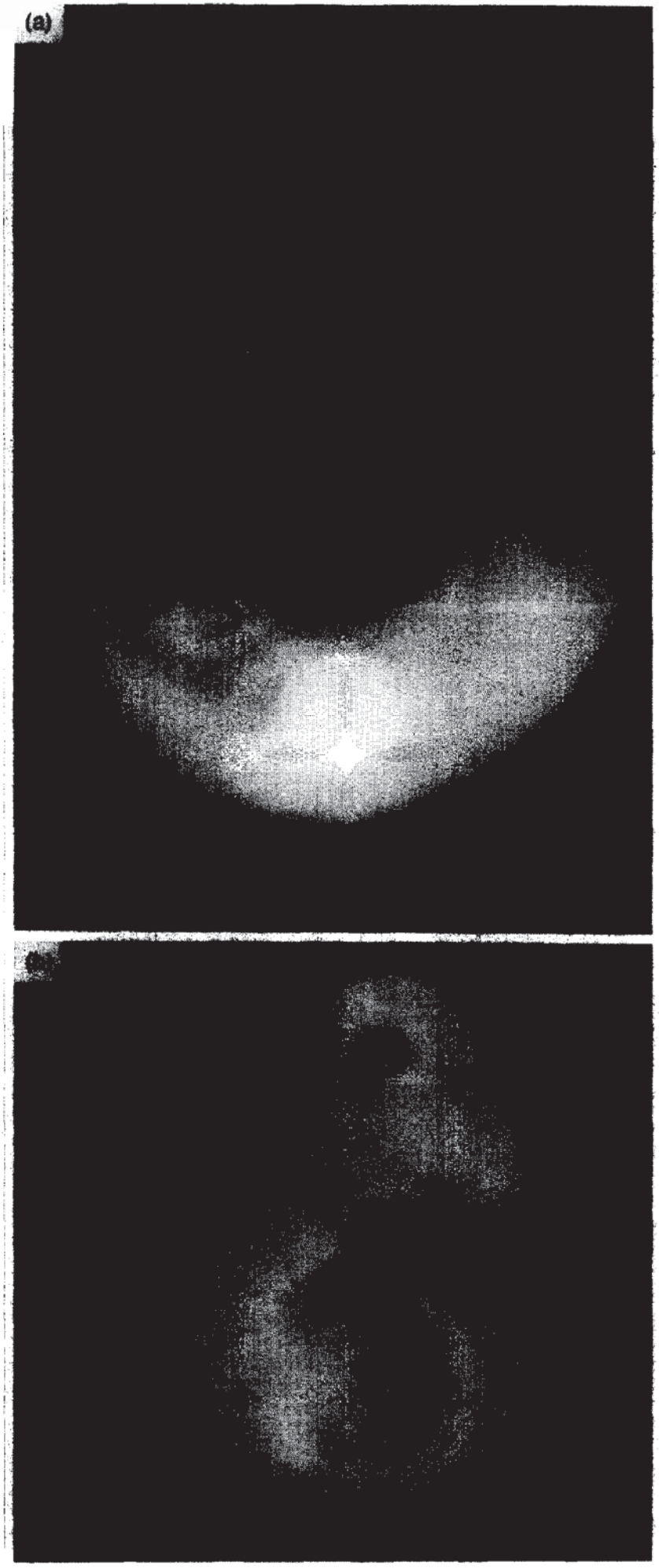

Plate 1. Rat embryos. (a) Control in normal human serum. (b) Embryo cultured in human PKU serum.

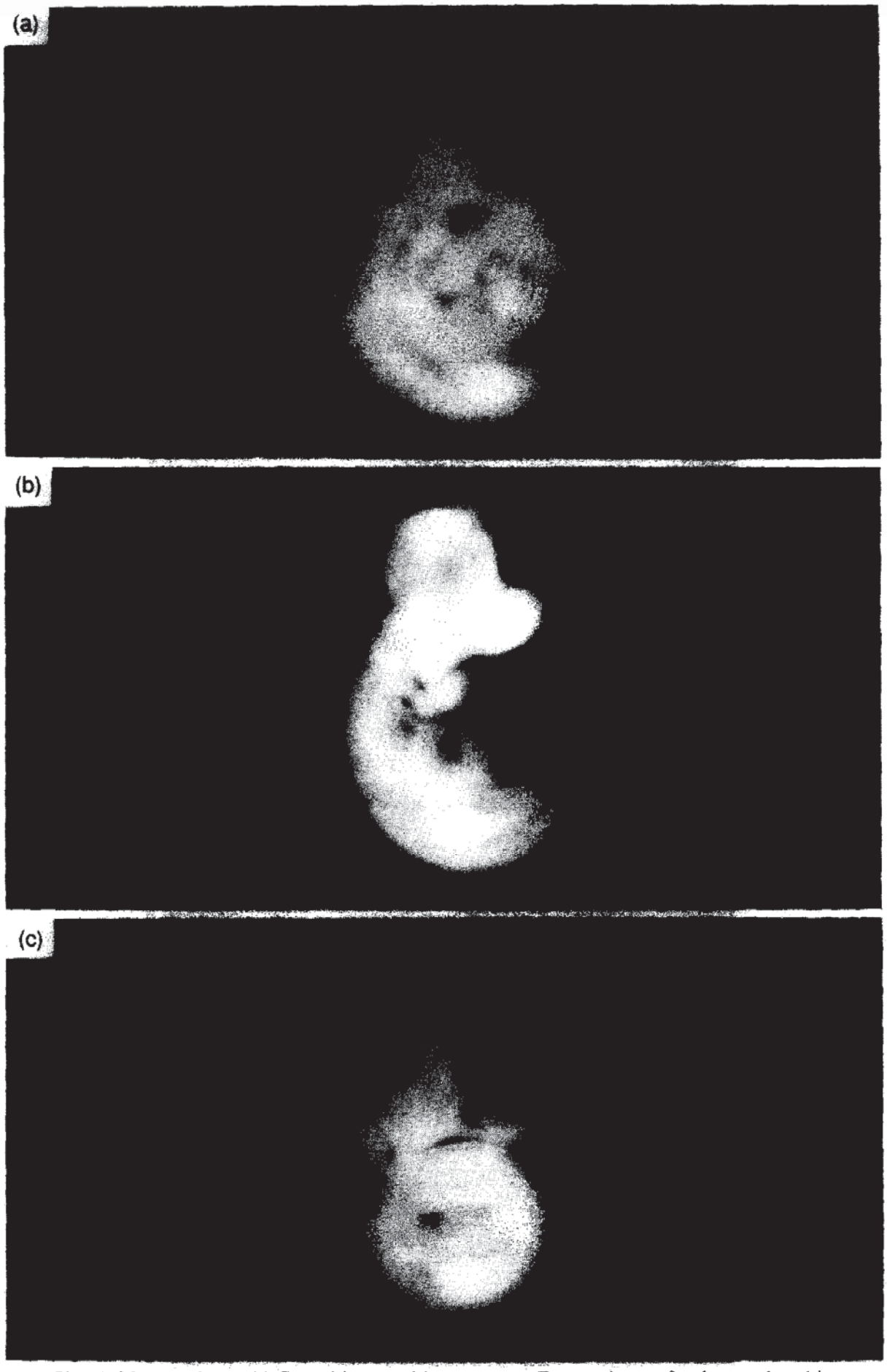

Plate 2. Mouse embryos. (a) Control in normal human serum. Two specimens of embryos cultured in human PKU serum showing (b) NTDs and (c) microcephalia.

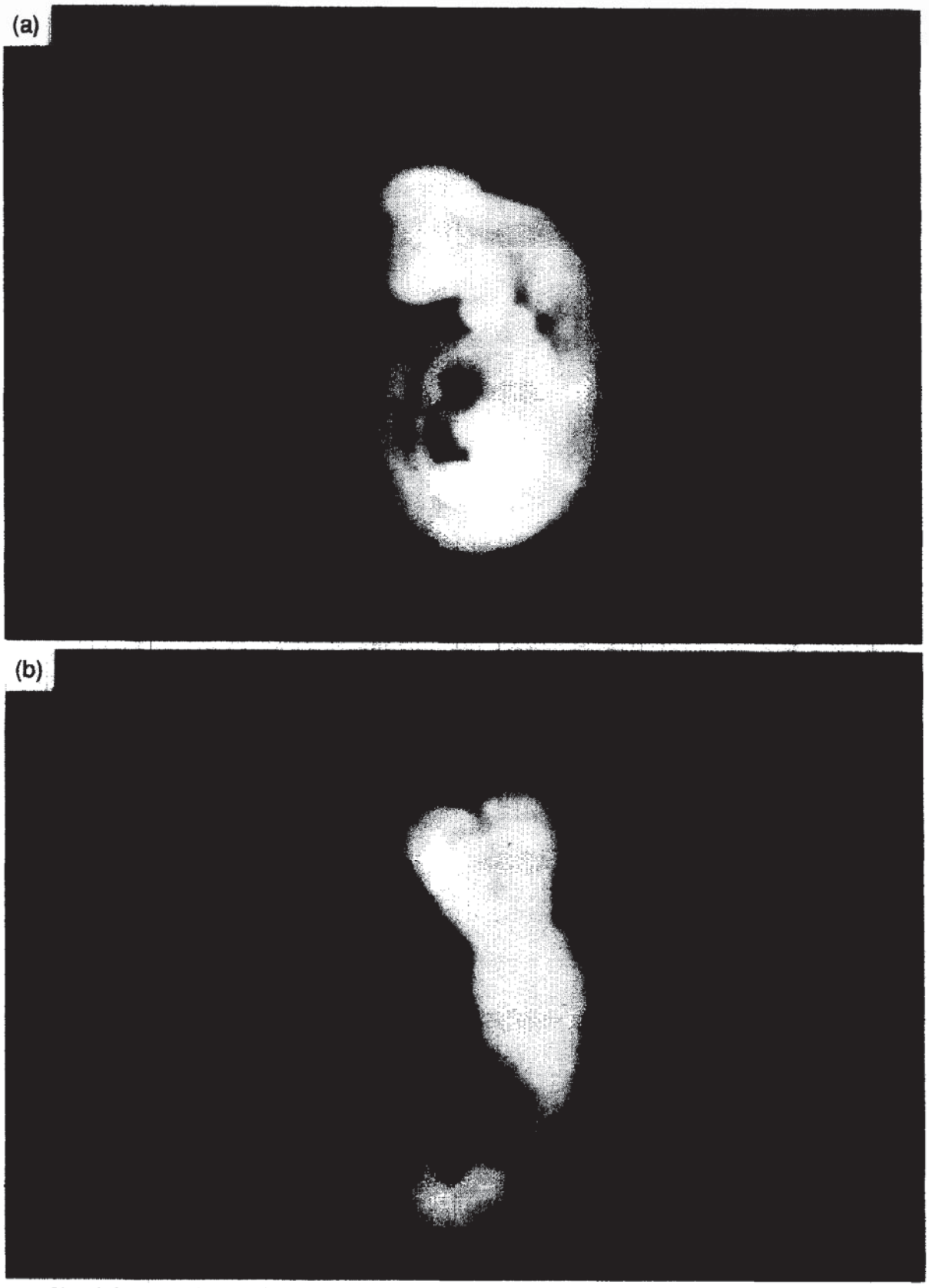

Plate 3. Mouse embryos cultured in human serum depleted of 5-HT and showing NTDs: (a) lateral view, (b) dorsal view.

male per cage). The embryos were taken from the uterus on the Monday of the following week, between 10.00 and 12.00 hr, so that their age was 10 days + 0–4 hr. We selected embryos that had about three somites and cultured them for 48 hr.

26 embryos in normal human serum and 10 embryos in rat serum were used as controls. 22 embryos were cultured in PKU serum.

In addition, we used mouse embryos, since they are generally considered to be more sensitive to teratogens than rat embryos. The mice we used were of the Swiss strain, provided by Iffa-Credo. They were housed in plastic cages, on a woodchip litter in groups of five and fed UAR AO3. The dark/light cycle was the same as for the rats. One human normal serum, for which the results were reproducible, was selected. In order regularly to obtain three-somite embryos, the animals were mated on Tuesday morning between 08.00 and 10.00 hr and the embryos removed on the Wednesday of the following week, at the age of 8 days + 4–6 hr.

20 embryos were examined as controls in rat serum. 47 embryos were cultured in normal human serum and 70 embryos were cultured in human PKU serum from 18 patients. Phe serum levels ranged from 7 to 27 mg/100 ml, and 5-HT levels ranged from 0.19 to 0.90 mg/litre.

Testing the hypothesis of a role for the deficiency of 5-HT

To test whether a deficiency of 5-HT plays a role in PKU embryopathy we used a medium consisting of normal human serum depleted of 5-HT to culture mouse whole postimplantation embryos. 5-HT was eliminated by passage on an ion-exchange column IRC 50. Growth factors and cytokines present in the serum were adjusted to the initial levels.

23 embryos were cultured in normal human serum as controls; 25 embryos were cultured in normal human serum depleted of 5-HT.

We first performed assays of 5-HT in tissues of embryos cultured either in normal or in PKU sera with the aim of investigating whether 5-HT is present in normal embryos and whether PKU serum can influence the results.

Biochemical analyses

Maternal plasma assays of Phe and 5-HT. Phe plasma concentrations were measured by fluorimetry. 5-HT was measured in serum by HPLC.

Embryonic tissue assays of 5-HT. After extraction with ethanol–acetone (1:1, v/v), non-conjugated 5-HT was measured by radioenzymology, according to the technique of Walker *et al.* (1983). To improve the specificity of this technique, the results of which are similar to those of the reference gas chromatography–mass spectrometry technique (Beck *et al.*, 1993), radiochromatography on silica-gel plates was performed.

Statistical analysis

Statistical comparisons were performed using either a chi-squared test or a rank test according to the nature of the data for consideration.

RESULTS

Rat embryo culture

There was no significant difference between controls cultured in rat or human serum with respect to embryonic development, as assessed by the developmental scoring system of Brown and Fabro (1981), or the number of somites after 48 hr of culture. The rates of malformed embryos were also comparable. Therefore it can be concluded that human serum is an adequate medium for rat whole embryo culture.

The data obtained by culture in human PKU serum led to a very definite and marked teratogenic action in this experimental protocol. All of the measured parameters (developmental score, number of somites, rate of malformed embryos) are significantly different from the controls. Malformations (Table 2; Plate 1) essentially concerned the branchial arches (hypoplasia), the telencephalon (hypoplasia), the 4th ventricle (enlarged and swollen or depressed), the body curvature (incomplete, irregular or even inversed), the somites (irregular), and the eyes (large optic vesicles with apparent enlarged pedicles, underdeveloped or absent lenses). These preliminary data were briefly reported previously (Roux *et al.*, 1991).

Mouse embryo culture

Mouse embryo culture in human PKU serum. The first observation was that the culture of mouse embryos in human serum is more difficult and less efficient than the culture of rat embryos. There was a clear-cut difference between embryos cultured in rat and normal human serum, the latter being

Table 2. Incidence of anomalies in rat embryos cultured in human control and PKU serum

Culture medium	Dead embryos	Developmental score (mean ± SD)	No. of somites (mean ± SD)	No. (%) of malformed embryos
Control rat serum (n = 10)		48 ± 0.91	32.38 ± 2.25	0
Control human serum (n = 26)	4	49.76 ± 2.32	32.95 ± 3.38	1 (3.8)
PKU human serum (n = 22)	1	45.80 ± 5.04**	29.95 ± 4.18*	21 (95.4)***

Values marked with superscripts differ significantly from the value for embryos cultured in control human serum: *$P < 0.05$ (Student's t-test); **$P < 0.01$ (Student's t-test); ***$P < 0.001$ (chi-squared test).

Table 3. Effects on three-somite mouse embryos of culture in control human serum and serum from PKU patients

	Development score	No. of somites	Yolk sac diameter (mm)	Head length (mm)†	Malformed embryos (%)	CNS anomalies (NTD, microcephalia)	Eye malformations	Body flexure anomalies
Control rat serum (n = 20)	46.04 ± 2.49	32.45 ± 2.11	4.5 ± 0.48	2.02 ± 0.29	0	—	—	—
Control human serum (n = 47)	43.02 ± 5.69^{ns}	31.41 ± 2.48^{ns}	4.52 ± 0.52^{ns}	1.81 ± 0.32	4 (8.5)^{ns}	4 (8.5)	4 (8.5)	0
PKU human serum (n = 70)	30.93 ± 6.11*	24.66 ± 4.24*	4.44 ± 0.79	1.53 ± 0.97	64 (91.4)**	54.10 (91.4)**	40 (57.14)**	22 (28.57)**

†*Head lengths were not* statistically tested, since the malformations essentially involved this region.
Values marked with asterisks differ significantly from the value for embryos cultured in control human serum (*$P < 0.001$, Student's *t*-test; **$P < 0.001$, chi-squared test); NS = not significantly different from embryos cultured in control rat serum.

delayed in their development: developmental scores and numbers of somites were lower at the end of the culture, but the differences were not statistically significant. Malformations were present in embryos cultured in normal human serum, but not in embryos cultured in rat serum; however, the difference was not statistically significant.

In comparison with embryos cultured in normal human serum, embryos cultured in human PKU serum were severely affected; all of their developmental parameters were significantly lower and they exhibited a very high rate of malformed specimens (96.3% in comparison with 8.5% in the controls). The malformations essentially concerned the central nervous system; most of the embryos had neural tube defects (NTDs). These NTDs involved the mesencephalon, and sometimes other parts of the encephalon. Some embryos had no NTD, but had a very small encephalon without any visible separation between the different cerebral vesicles and with a smooth and translucent wall. Eye abnormalities were also very frequent (micro- or anophthalmia), but they were not easy to detect when an NTD was present. Therefore only the obvious ones were recorded, and their rate is underestimated. Also very characteristic were the somite irregularities and the abnormalities of body flexure which were generally associated (Table 3; Plate 2). It should be emphasized that the malformed controls presented exclusively NTD or microcephalia; the rest of their body was normal.

Therefore we consider that these results established that post-implantation whole rat or mouse embryo culture is an appropriate model for the study of PKU embryopathy. Since mouse embryos are definitely more sensitive than rat embryos, we used mouse embryos for the subsequent experiments.

Testing the serotonin hypothesis

Serotonin concentration. In a preliminary experiment, we performed serotonin assays on whole mouse embryos at the end of the 48-hr culture in normal human serum (control embryos) or in PKU media (treated embryos). Serotonin was detected in all six control embryos. The levels, expressed as pmol/mg of protein, were 10, 20, 24, 93, 117, 129. Serotonin was not detected in any of six malformed treated embryos.

Table 4. Effects on mouse embryos of culture in normal human serum depleted of 5-HT

	Controls (normal human serum)	Treated (5-HT-depleted serum)
No. of embryos	23	25
No. of malformed embryos	5	25*
No. (%) of embryos with malformations:		
NTD	5 (21.7%)	25 (100%)
Eye	5	25
Somites, body flexure	1 (4.34%)	9 (60%)*

Values marked with an asterisk differ significantly (chi-squared test) from the value for embryos cultured in control serum (*$P < 0.001$).

Effect of 5-HT-depleted human serum. Subsequently, we used either normal human serum or 5-HT-depleted human serum (from non-PKU donors) to culture mouse embryos. The rate of malformed control embryos was rather high in this group (20% of NTD and eye abnormalities) but not significantly different from that in the controls in the previous experiment. The difference between control and 5-HT-depleted embryos was very obvious, since 100% of the latter were malformed ($P < 0.001$, chi-squared test). Again, the treated malformed embryos were much more severely affected (NTD and eye abnormalities, irregularities of somites and perturbations of body flexure) some of them being monstrous (Table 4; Plate 3).

DISCUSSION AND CONCLUSION

Several interpretations of PKU embryopathy have been proposed and all are based on the hypothesis of the teratogenicity of direct metabolic abnormalities (hyperphenylalaninaemia and/or formation of abnormal Phe metabolites).

A few authors have tested the effect of hyperphenylalaninaemia, experimentally induced by ingestion of excessive amounts of Phe, in genetically normal animals. They studied either mouse foetuses (Yamawaki *et al.*, 1988) or rat (Lacy, 1986) or mouse (Sasahara *et al.*, 1987) pups brain development and observed some abnormalities. However, hyperphenylalaninaemia must be considered as only one element of a very complex metabolic dysfunction. This excessive intake of Phe is not physiological and does not reflect the PKU disease. The possible metabolic consequences of hyperphenylalaninaemia should be taken into account.

The use of whole rat or mouse embryo culture has the obvious advantage of allowing us to choose the environmental medium of the embryo and, therefore, to analyse the possible consequences of different parameters on embryonic development during a very sensitive period. This technique has been used by several authors to study the possible embryotoxicity of Phe and its different metabolites that exist in PKU.

Hamers *et al.* (1989) cultured rat embryos in rat serum to which tested compounds were added. In this system Phe was not embryotoxic. Phenylpyruvic acid induced some growth retardation whereas phenyllactic acid did not. Hydroxyphenylacetic acid and phenylacetic acid induced a dose-related embryotoxicity.

Denno and Sadler (1990) cultured mouse embryos by a similar technique. Their results showed a dose-related embryotoxicity of Phe and its PKU metabolites (phenylethylamine, phenylpyruvic acid, phenylacetic acid, 2-hydroxy phenylacetic acid and phenyllactic acid). The embryotoxicity differed according to the metabolite, phenylethylamine being the most active.

However, it must be emphasized that the concentrations of metabolites used in these experiments were always higher than those observed in the serum of PKU patients. In fact in such patients these metabolites are rapidly eliminated in the urine.

A mouse mutant deficient in PAH was described in 1990 by McDonald *et al.*, but its features definitely differ from the human disease: it is necessary to overdose the animals with Phe to observe a hyperphenylalaninaemia and there is no gross pathology in homozygotes. As far as we know, no information is available about the progeny of these mice.

Thus we decided (i) to use human PKU serum as a medium for whole embryo culture to mimic clinical conditions as closely as possible, and (ii) to test the hypothesis of PKU embryotoxicity as a consequence of competitive inhibition of aromatic amino acid hydroxylases. Indeed some previous data support this hypothesis.

In 1972 Kirby and Gilmore showed that the notochord of early chick embryo (presympathetic stage) is a 'possible site of catecholamine synthesis and storage'. It can therefore be hypothesized that catecholamines could behave as morphogens in early embryonic development.

With regard to serotonin, in 1988 Lauder *et al.* showed that 'the presence of 5-HT uptake sites in epithelia and adjacent sites of serotonin binding proteins in the underlying mesenchyme raises the possibility that 5-HT might be involved in those epithelial–mesenchymal interactions known to be important for the development of structures in the cranio-facial region' [in gestation day (GD) 12 embryos]. In 1992, Shuey *et al.*, by exposure of mouse embryos to sertraline (an inhibitor of 5-HT uptake) in the critical period of GD 10–11, demonstrated that 'it appears that inhibition of 5-HT uptake into cranio-facial epithalia may produce developmental defects by interference with serotoninergic regulation of epithelial mesenchymal interactions important for normal cranio-facial development'.

In the first part of this study, we defined an animal model for PKU embryopathy, in rats and mice, the latter species being the more sensitive. The use of PKU human serum affords the advantage of allowing experimental embryos to develop in a milieu very similar to that of human embryos of PKU pregnant women. This model should prove useful in testing different hypotheses.

Our second aim was to test the 'serotonin hypothesis'. Our preliminary data indicate that serotonin is present in mouse embryos cultured in normal human serum, but is undetectable in abnormal embryos cultured in human PKU serum which is known to have a low level of serotonin. These results can only be considered as suggesting a possible role for serotonin in embryonic development. At least they indicate that serotonin is present very early in normal embryos.

We also showed that normal human serum depleted of 5-HT has teratogenic effects similar to those of PKU serum. This finding supports the 'serotonin hypothesis'.

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