

Lacticacidaemia due to pyruvate dehydrogenase deficiency, with evidence of protein polymorphism in the α -subunit of the enzyme

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Abstract. In three infants with neonatal lacticacidaemia, a deficiency in the E_1 (pyruvate dehydrogenase) component of the pyruvate dehydrogenase complex was demonstrated in skin fibroblast cultures. Residual activities of the pyruvate dehydrogenase complex in the activated state were 1.6%, 3.9% and 18.8% of control values, respectively. Immunoprecipitation of extracts of cultures skin fibroblasts grown on ³⁵S-methionine with anti-pyruvate dehydrogenase complex antibody revealed an abnormality in the $E_1\alpha$ -component of these three patients when visualised after sodium dodecyl sulphate/polyacrylamide gel electrophoresis. This component appeared to have a slightly lower molecular weight than did this protein from control cell strains. Cell strains from other patients with a deficiency of the pyruvate dehydrogenase complex did not exhibit this defect. Three patients also showed dysmorphism and developmental abnormalities of the central nervous system.

Key words: Lacticacidaemia – Pyruvate dehydrogenase deficiency – Protein polymorphism – Developmental abnormalities

Introduction

The pyruvate dehydrogenase component (E_1) carries out the first step, namely the oxidative decarboxylation of pyruvate in the reaction catalysed by the pyruvate dehydrogenase complex (PDHC) (Fig. 1). Reports of pyruvate dehydrogenase (E_1) deficiency have described children either with ataxic episodes [3–5, 16] or with delayed motor and neurological development [8, 9, 16, 17, 18]. Other types of presentation resembled Leigh's encephalomyelopathy [2, 7, 20] or were characterised by overwhelming lacticacidemia in the neonatal

period [9, 16, 19]. We have described 18 patients with pyruvate dehydrogenase (E_1) deficiency [14]. One major finding was facial dysmorphism or microcephaly in ten of them. Here we describe three cases of severe pyruvate dehydrogenase deficiency with evidence of protein polymorphism in the $E_1\alpha$ component.

Materials and methods

Cultures of skin fibroblasts. Skin fibroblast cultures from the patients and age-matched controls were grown in α -minimal essential medium (α -MEM) supplemented with 10% fetal calf serum. α -MEM contains 0.5 mM pyruvate as one of its growth constituents.

Measurement of enzyme activities in cultures skin fibroblasts. Pyruvate dehydrogenase complex activity was measured in the native (unstimulated) and dichloroacetate activated state by the method of Sheu et al. [17] modified as suggested by Hyland and Leonard [10]. Pyruvate dehydrogenase (E_1) was measured as described in [3]. Dihydrolipoyl transacetylase was measured by the method of Butterworth et al. [6] and dihydrolipoyl dehydrogenase by the method of Reed and Wilms [13]. Pyruvate carboxylase was measured by the method of Ballard and Hanson [1] and whole cell pyruvate oxidation as described [16].

Preparation of antisera. Rabbit antiserum against the purified pyruvate dehydrogenase complex isolated from pig heart [11] was prepared by an intramuscular injection followed by subcutaneous injections at weekly intervals. Pre-immune sera were collected and used as normal rabbit sera. Anti-pyruvate dehydrogenase complex antiserum was shown to inhibit pyruvate dehydrogenase complex activity in solution. It was also shown to immunoprecipitate the antigens $E_1\alpha$, $E_1\beta$ and the E_2 transacetylase when added to the pyruvate dehydrogenase complex.

Immunoprecipitation of ³⁵S-methionine labelled proteins. Cultured skin fibroblasts were grown to early confluence in 75 cm² tissue culture flasks, one per cell strain per experiment. One day prior to radioactive labelling, the medium was re-

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Abbreviations: PDHC = pyruvate dehydrogenase complex;
 α -MEM = α -minimal essential medium

moved from the flasks and replaced by 10 ml α -MEM medium containing 10 μ M methionine. The following day the medium was replaced by 5 ml of the same medium supplemented with 200 μ Ci/ml of 35 S-methionine (800–1,200 Ci/mmol, Amersham, Arlington Heights, Ill.). The cells were incubated overnight and the radioactive medium was removed. Cells were extracted as described by Robinson et al. [15], first with Triton X-100 and then with carbon tetrachloride. The resulting solution was subjected to an immunoprecipitation sequence with normal rabbit serum before immunoprecipitation with 10 μ l pyruvate dehydrogenase antibody in 1 ml, 10 mM Tris-HCl pH 7.4, 5 mM phenylmethyl sulphonyl fluoride, 1 mM EDTA, 0.1% Triton X-100 and 1 μ g/ml methionine. Immunoprecipitates were pelleted by the addition of IGsorb (The Enzyme Centre, Waltham, Mass.). The resulting pellets were taken up into a 1% sodium dodecyl sulphate solution for electrophoresis on 10% polyacrylamide slab gels.

35 S-labelled protein bands were visualised by fluorography with an exposure time of 72 h.

Case reports

Case 1

A girl was born to a 26-year-old G₁P₀ mother by Caesarian section at 42 weeks. The mother was treated by sulphasalazine for Crohn's disease. Apgar scores were 2 at 1 min and 3 at 5 min. She required 45 min artificial ventilation. Initial metabolic acidosis was treated with sodium bicarbonate. Physical examination demonstrated an elfin face and a blowing systolic ejection murmur at the lower left sternal border. She had short arms and legs and bilateral simian creases. On day 5, slow tonic clonic seizures were noted and treated by phenobarbital. Throughout the first 40 days of life, she had episodic metabolic acidosis requiring intravenous bicarbonate administration. Blood lactic acid was 18.3 mM. At 30 days of age blood bicarbonate was 11 mEq/l despite dosage with 15 mEq bicarbonate/day. She became increasingly lethargic and required ventilator support to avoid respiratory arrest.

At 40 days of age she was transferred for evaluation. Her weight was 2.94 kg, and length 49 cm. She was flaccid, pale and very hypotonic. She was breathing spontaneously but required a back-up ventilator rate of 15. Her corneal reflexes were absent on the right, and intermittently present on the left. She had episodic seizures. A clinical diagnosis of valvular aortic stenosis was made.

On admission, serum sodium was 136 mEq/l, potassium 2.4 mEq/l, chloride 92 mEq/l, bicarbonate 17 mEq/l, glucose 118 mg/dl, creatinine 2.6 mg/dl, calcium 9.4 mg/dl. Blood ammonia was 110 μ M/l, lactate 18.6 mM/l (normal 0.5–2.2) and pyruvic acid 0.43 mM/l (normal < 0.15 mM/l). Other organic acids were normal.

She required increasing amounts of bicarbonate (44 mEq on the third day) to maintain an acceptable blood pH. Biotin (10 mg) per oral-gastric tube did not cause clinical improvement. A CAT scan demonstrated extensive hydrocephalus and loss of brain tissue. An EEG revealed slow wave activity with intermittent spikes. On the third hospital day she was given 5 mg thiamine per oral-gastric tube. During the next 24 h, she became alkalotic with a pH of 7.62 and a bicarbonate level of 23 mEq/l. Bicarbonate therapy was discontinued and over the next 24 h she maintained a normal pH. However, she

continued to require artificial ventilation. In view of the grossly abnormal CAT scan results, the patient was removed from the ventilator and expired on the 44th day of life. Autopsy showed hydrocephalus with polymicrogyria, widespread past and recent necrosis of the cerebral hemisphere and brain stem, ectopic olivary nuclei, absent corpus callosum, and aortic valvular stenosis.

Case 2

A girl was hospitalised at 6 days of age following a series of brief apnoeic episodes at home. Her parents are unrelated. Her newborn nursery course and first days at home had been unremarkable. Physical examination revealed a lethargic, hypotonic infant with periods of apnoea accompanied by bradycardia, lasting 20–40 s; both responded to bagging. Focal seizure activity (twitching of the right hand and foot) was noted. Initial laboratory studies included normal serum electrolytes, T4, TSH, blood count, glucose, calcium, magnesium and arterial blood gas determinations; serum transaminases and blood ammonia were at slightly elevated levels. After finding elevated urine and plasma alanine levels, markedly increased blood lactate (8.4 mmol/l) and pyruvate (0.66 mmol/l) were documented. Urinary organic acid analysis showed elevations of lactate, pyruvate, and alpha-ketoglutarate. Ultrasound examination of the cranium showed mild dilation of the lateral and third ventricles. Thiamine triphosphate synthetase inhibitor was absent from urine (courtesy of Dr. Jack Cooper, Yale University). An EEG at the age of 7 months showed a multifocal spike pattern.

A brief trial with oral lipoic acid and thiamine was not followed by clinical or biochemical improvement. At the age of 22 months, the patient shows marked growth retardation and developmental delay; her developmental age is 5–8 months with cognitive skills being somewhat higher than motor skills. She has an unusual facial appearance with prominence of the frontal region, bitemporal narrowing, a short, upturned nose, full cheeks, and a wide mouth. There is hypotonia and slight hyperflexia. Although she has had no further episodes of apnoea, she continues to have elevated levels of blood lactate (5.7–8.5 mmol/l) and pyruvate (0.52 mmol/l).

Case 3

This girl was the first-born of unrelated Caucasian parents. The 32-year-old, gravida 2, para 1 mother entered spontaneous labour and breech delivery at term following an uneventful pregnancy. The amniotic fluid was meconium-stained. The 2510 g infant had an Apgar score of 2 at 1 min. Immediate bagging and intubation were required because of inadequate respiratory effort. By 2 h of age, oxygenation was adequate with spontaneous ventilation in room air.

On physical examination the patient was found to be lethargic, hypotonic, areflexic and inactive. Respiration was rapid. Eye movements were minimal. Her head circumference was 34.2 cm (60th percentile) and crown-heel length 48 cm (30th percentile). The fontanelle was soft. There was no clinical or laboratory evidence of cardiopulmonary disease. There was micrognathia, low set ears with normal pinnae, narrow palpebral fissures, short neck with redundant skin, thick, "straight" shocky hair, genua valga, joint contractures at the elbows, shoulders and hips and pes cavus with hammer toes. The cry was weak and root, suck and gag reflexes were absent.

Table 1. Results of enzyme analysis

	1373 Patient 1	1558 Patient 2	1585 Patient 3	Controls
nmol/min per mg protein				
PDH (native)	0.015 ± 0.008 (9)	0.100 ± 0.028 (9)	0.034 ± 0.020 (7)	0.582 ± 0.080 (7) (0.332 – 0.668)
PDH (DCA activated)	0.012 ± 0.008 (9)	0.141 ± 0.044 (8)	0.029 ± 0.010 (7)	0.749 ± 0.071 (7) (0.542 – 0.868)
nmol/min per mg				
E ₁ Pyruvate dehydrogenase	0.322 ± 0.278 (4)	0.486 ± 0.188 (7)	0.492 ± 0.173 (5)	1.37 ± 0.205 (7) (0.7 – 3.5)
E ₂ Dihydrolipoyl transacetylase	16.8 ± 3.5 (3)	25.0 (1)	17.7 (1)	21.5 ± 3.0 (10) (9.7 – 36.0)
E ₃ Dihydrolipoyl dehydrogenase	14.2 ± 2.3 (4)	7.2 ± 2.1 (2)	10.9 (1)	6.9 ± 1.0 (9)
Pyruvate carboxylase	2.1 ± 0.7 (4)	3.46 (1)	0.83 (1)	3.2 ± 0.81 (5)
Whole cell 1 ⁻¹⁴ C-pyruvate oxidation	4.9 ± 1.7 (5)	9.03 ± 2.1 (5)	4.5 ± 1.6 (4)	22.2 ± 4.4 (5) (15.7 – 32.6)

Values are mean ± S.E. (number of determinations in parenthesis)

Values are given for controls performed on the same day as the cell strain to be tested together with the range of those values

DCA = dichloroacetate

She did not follow a light. Her head lag was marked, and there was no trunk or leg supporting response. The Moro reflex consisted of minimal arm extension. Deep tendon reflexes were absent. There was no hepatosplenomegaly.

There was no clinical or biochemical change following the oral administration of thiamine (1 g/day) over 3 months.

The initial investigation revealed a persistent metabolic acidosis with an elevated anion gap (pH 7.35, PCO₂ 29, bicarbonate 16 mEq/l, sodium 138 mEq/l, potassium 4.0 mEq/l, chloride 106 mEq/l). The blood lactic acid concentration was 21 mmol/l and pyruvic acid concentration 0.38 mmol/l. Initial serum ammonia was 176 μmol/l (normal less than 110), but this fell to 85 μmol/l within 1 day. Serum amino acids, glucose, electrolytes, SGOT, T₄ and TSH were normal. Chromosomes were normal. There was no inhibitor of thiamine triphosphate synthetase in the urine. Marked elevation of urinary lactic acid and alpha-ketoglutaric acid was found with a slight elevation of malic acid and fumaric acid. Muscle biopsy showed occasional enlarged type I fibres, but no "ragged-red" fibres were present. Electron microscopy of muscle gave normal results. A CT scan on the first day showed moderate ventricular enlargement with extensive hypodensity of white matter.

Results and discussion

Activities of the enzymes of pyruvate metabolism

The activity of the pyruvate dehydrogenase complex was measured in cultured skin fibroblasts in the native state and after activation with dichloroacetate (Table 1). The activities of the enzyme complex in the native state were 2.6%, 5.8% and 17.2% of the control values, respectively while in the activated state the values were 1.6%, 3.9% and 18.8%, respectively. There was thus, no significant activation with dichloroacetate. The assay system of Sheu et al. [17] in our hands only gives a 50% increase in activity of the pyruvate

dehydrogenase complex after activation with dichloroacetate. This we believe is due to the fact that our cells are cultured in α-MEM, a pyruvate-containing culture medium. Assay of the three catalytic components of the complex revealed decreased activity of pyruvate dehydrogenase (E₁), rather than of dihydrolipoyl transacetylase (E₂) or of dihydrolipoyl dehydrogenase (E₃). The higher residual activities of the pyruvate dehydrogenase component, as compared to those of the pyruvate dehydrogenase complex, lead us to suggest that the former assay measures some other enzyme activity that decarboxylates pyruvate or that the defects in E₁ activity are more deleterious to the activity of the whole complex than to the

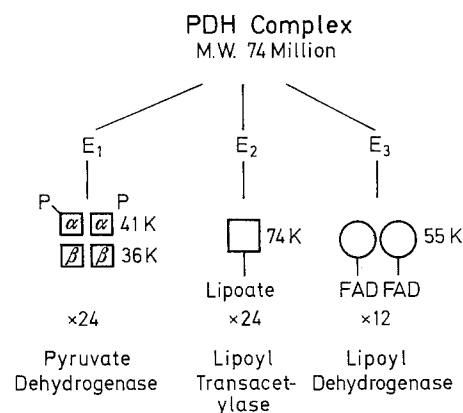


Fig. 1. Composition of the pyruvate dehydrogenase complex in bovine kidney (after Reed [12]). The total complex has a molecular weight of 7.4×10^6 daltons, being made up of three catalytic components. The E₁, dehydrogenase is made up of 24 α₂β₂ tetramers, the α-subunit of which can be phosphorylated and dephosphorylated. The E₂ lipoyl transacetylase is made up of 24 monomers of 52 kilodaltons, which appear to be 74 kilodaltons by polyacrylamide gel electrophoresis and have covalently attached lipoic acid. The E₃ lipoyl dehydrogenase is made up of 12 dimers of 55 kilodaltons which have tight binding sites for flavin adenine dinucleotide (FAD)

isolated E_1 activity, measured with artificial electron acceptors ($\text{Fe}(\text{CN})_6^{3-}$). The activity of PDHC correlates well with the rate of $1\text{-}^{14}\text{C}$ -pyruvate oxidation in whole cells. It might be postulated because of the lack of activation seen in the pyruvate dehydrogenase complex of these patients that the low activity of the complex was due to pyruvate dehydrogenase phosphatase deficiency. Though we cannot eliminate this possibility totally, it is unlikely because of the polymorphism in the $E_{1\alpha}$ pyruvate dehydrogenase component demonstrated in the following section.

Immunoprecipitation of the pyruvate dehydrogenase complex

Skin fibroblasts were cultured in a medium containing ^{35}S -methionine to label newly synthesised proteins and the CCl_4 cell extracts incubated with anti-pyruvate dehydrogenase antiserum. The immunoprecipitates formed both with this antiserum and control pre-immune serum were electrophoresed on 12.5% polyacrylamide/sodium dodecyl sulphate gels and the resulting labelled protein distribution was examined by

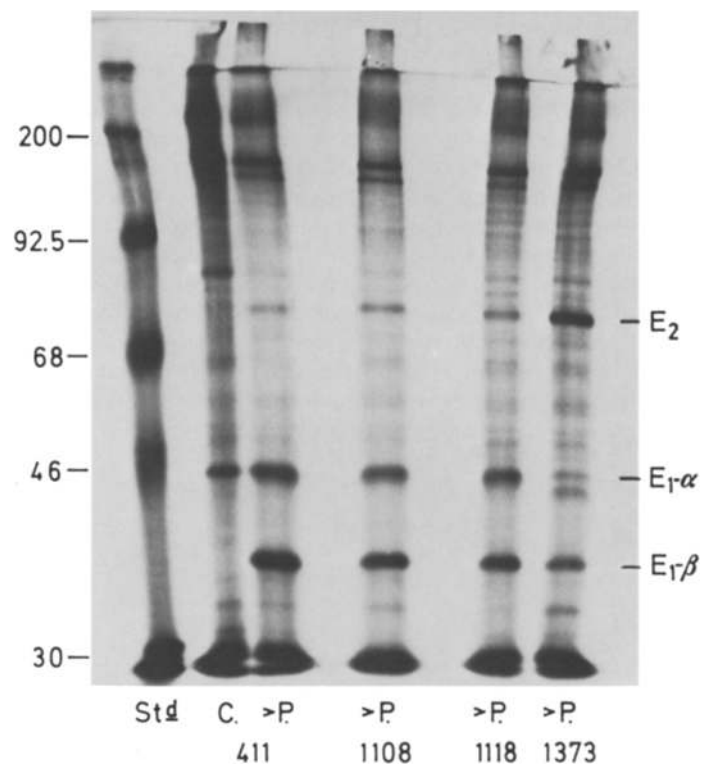


Fig. 2. Protein polymorphism in pyruvate dehydrogenase-deficient cell lines. Proteins were immunoprecipitated from ^{35}S -methionine labelled cultured skin fibroblast extracts with either control serum (C) or anti-pyruvate dehydrogenase antiserum (P). The immunoprecipitates were run on standard sodium dodecyl sulphate/12.5% polyacrylamide electrophoretic gel and the pattern of radioactivity revealed by fluorography. Lanes from left to right are *lane 1*, protein standards (myosin $M_r = 200000$, phosphorylase b, $M_r = 92500$, albumin $M_r = 68000$, ovalbumin $M_r = 46000$, carbonic anhydrase $M_r = 30000$); *lane 2*, control cell line 411 with control serum; *lane 3* control cell line 411 with anti-PDH anti-serum; *lane 4*, PDH-deficient cell line 1108 with anti-PDH antiserum; *lane 5*, PDH-deficient cell line 1118 with anti-PDH antiserum; *lane 6*, PDH-deficient cell line 1373 with anti-PDH antiserum. $E_{1\alpha}$, $E_{1\beta}$ indicate the positions of the α and β subunits of the E_1 pyruvate dehydrogenase while E_2 indicates the position of the dihydrolipoyl transacetylase subunit from the PDH complex

fluorography (Fig. 1). The pattern with anti-pyruvate dehydrogenase antiserum revealed the presence of dark bands corresponding to proteins at 74 K, 43 K, and 36 K. These correspond to the positions of E_2 , $E_{1\alpha}$ and $E_{1\beta}$ respectively (Fig. 2), all members of the pyruvate dehydrogenase complex except that the $E_{1\alpha}$ component of bovine kidney is slightly lower at 41 K [12]. The antibody was not found to have activity towards E_3 either by this technique or by Ouchterlony diffusion. Control serum brings down a major band at 43 K which can be reduced in intensity by prior pre-incubation and immunoprecipitation with a monoclonal antibody against actin.

Two cell lines (1108 and 1118) with PDHC deficiency activities similar to 1558 (from patient 2) show the same fluorographic pattern as the control cell line 411 (Fig. 1). However, the deficient cell line 1373 (from patient 1) shows a split double band corresponding to $E_{1\alpha}$: One of the bands is in the same position as actin (43 K) or the control $E_{1\alpha}$ band while the other appears to be somewhat smaller (41 K). When a monoclonal antibody against actin was used to remove actin, 1373

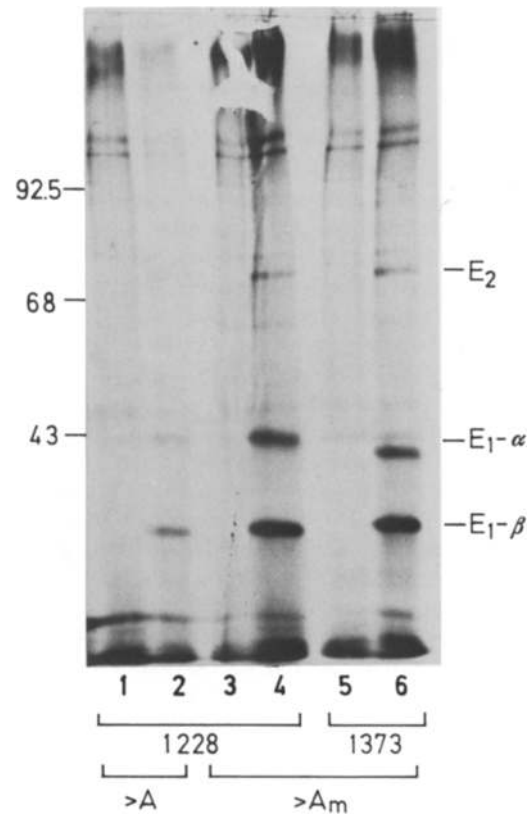


Fig. 3. Immunoprecipitated ^{35}S -labelled proteins obtained with anti-pyruvate dehydrogenase antiserum after treatment of the extracts with anti-actin antiserum. Experiments were performed as described for Fig. 2 except that two samples were pre-extracted with $10\ \mu\text{l}$ anti-actin (anti-A) (Miles-monospecific) and four samples with $10\ \mu\text{l}$ anti-actin antiserum (anti-Am) (Amersham monoclonal). *Lane 1*, control cell line 1228 pre-immunoprecipitated by anti-A then by control serum; *lane 2*, control cell strain 1228 pre-immunoprecipitated by anti-A followed by anti-PDH antiserum; *lane 3*, control cell strain 1228 pre-immunoprecipitated with anti-Am followed by control serum; *lane 4* control cell strain 1228 pre-immunoprecipitated with anti-Am followed by anti-PDH antiserum; *lane 5*, PDH-deficient cell line 1373 pre-immunoprecipitated with anti-Am followed by control serum; *lane 6*, PDH deficient cell line 1373 pre-immunoprecipitated with anti-Am followed by anti-PDH antiserum

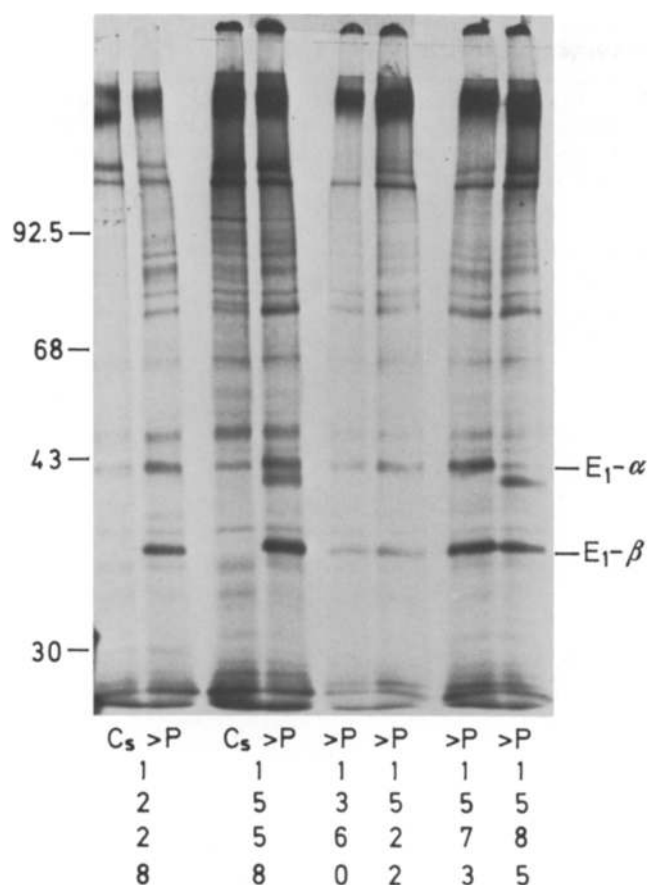


Fig. 4. Protein polymorphism in pyruvate dehydrogenase deficient cell lines. Proteins were immunoprecipitated from ^{35}S -methionine labeled cultured skin fibroblast extracts with either control serum (C_s) or anti-pyruvate dehydrogenase antiserum (P). The immunoprecipitates were run on standard sodium dodecyl sulphate/12.5% polyacrylamide electrophoretic gel and the pattern of radioactivity revealed by fluorography. Lanes from left to right are: *lane 1*, control cell line 1228 with control serum; *lane 2*, control cell strain 1228 with anti-PDH antiserum; *lane 3*, PDH-deficient cell strain 1558 with control serum; *lane 4*, 1558 with anti-PDH antiserum; *lane 5, 6, 7, 8*, PDH-deficient cell strains 1360, 1552, 1573 and 1585 with anti-pyruvate dehydrogenase antiserum. $E_1\alpha$ and $E_1\beta$ indicate the position of α and β subunits of the E_1 pyruvate dehydrogenase component

showed only a predominant 41 K band (Fig. 3). When monospecific antibody to actin was used it did not appear to remove the contaminating actin band as effectively with preservation of the PDH-specific antigens. The two other PDHC-deficient cell lines 1585 (patient 3) and 1558 (patient 2) showed the double band demonstrated in 1373, which was not seen in ten other PDHC-deficient cell lines tested nor in any control cell line tested (Fig. 4).

Despite the fact that these three children had the same basic enzyme defect, they showed some differences. Patient 2 had facial dysmorphism with microcephaly, patient 3 had less pronounced features of dysmorphism and patient 1 had a normal appearance. Two of the children had increased α -keto-glutarate excretion in the urine. All three had lacticacidaemia, difficult to control and (patient 1) died in the neonatal period. In this child, the brain was already morphologically affected at birth, showing agenesis of the corpus callosum and ectopic olivary nuclei. The brain stem showed cystic lesions at autopsy. A CT scan revealed hypodense white matter in patient 3

with moderate ventricular enlargement, while patient 2 had microcephaly and ventricular dilation noted by ultrasound.

Pyruvate dehydrogenase deficiency appears to effect the development of the head so that either malformation of the brain, microcephaly and/or facial dysmorphism may occur. Subsequently, cystic lesions may develop in the white and gray matter, especially in the basal ganglia. Since the brain stem is perhaps the most metabolically active area, areas of necrosis indicate that cell death is occurring. This could be due either to lack of intracellular ATP or to locally high concentrations of lactic acid secondary to the enzymatic deficiency.

In summary, we present evidence for protein polymorphism of the E_1 component in three patients with PDH- E_1 deficiency.

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SYNDROMES are the signpost and compass and map which direct us through the crowded twisting streets, the oceans, and the jungles of medical experience. They are not points of arrival, nor things. They are not immutable, and are always to be reviewed critically. A full understanding of the logical system implied by the syndrome should enable us to guide our patient with confidence and wisdom; should suggest and direct our researches; and should refresh our sense of gratitude to our medical forefathers.

William Goody, *Lancet* 1:1, 1961