Biochemical Changes in Cultured Foetal Rat Liver Explants

By PAUL C. MACDONNELL,* ELENA RYDER,† JUAN A. DELVALLE and
OLGA GREENGARD

*Department of Biological Chemistry, Harvard Medical School, and
the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass. 02215, U.S.A.*

(Received 21 February 1975)

Liver explants from 20-day-old fetuses cultured for 48 h in the absence of serum released 70% of their total soluble protein content into the medium. In the presence of serum this loss still amounted to 60%. The concentration of total particulate protein remained unchanged but there was some translocation of mitochondrial enzymes to the cytosol, and enzymes expected to increase during this stage of development failed to do so. The addition of cortisol plus glucagon (to serum-containing media) did not decrease the loss of total soluble protein from the explants but induced considerable tyrosine aminotransferase activity which was not released into the medium. The observations suggest that under the usual culture conditions a minority of the cells retain their functional integrity. The extent of deterioration, not reflected in histologically visible necrosis or cell damage, can be conveniently monitored by the malate dehydrogenase activity released to the medium.

During the last few days of gestation the enzymic composition of foetal rat liver undergoes considerable change (Greengard, 1971). Explants of such livers *in vitro*, if they were 'viable' for some 48 h, would provide a good system for (a) testing the extent to which biochemical differentiation can continue in the absence of extrahepatic foetal or maternal factors and for (b) attempting to identify such factors. The definition of 'viability' is, however, a major problem. For example, explants are capable of incorporating precursors into protein and RNA (Wicks, 1968; Sereni & Sereni, 1970; Rih *et al.*, 1971) but these synthetic capacities have not been quantitatively compared with those *in vivo*. The preferential accumulation or loss of particular enzymes that are known to increase or diminish during this period *in vivo* may provide more specific criteria for continued differentiation. The only study (Tourian, 1973) that attempted this type of comparison yielded a negative result: the activity of phenylalanine hydroxylase (EC 1.14.16.1) diminished during culturing, whereas *in vivo* it is known to increase during foetal life. On the other hand, the study of two enzymes that are absent from normal foetal liver, tyrosine aminotransferase (EC 2.6.1.5) and phosphoenolpyruvate carboxykinase (EC 4.1.1.49), yielded positive results: these enzymes, which can be prematurely evoked *in utero* (with glucagon) (Greengard & Dewey, 1967;

Yeung & Oliver, 1968), could also be evoked in the foetal liver explants (with the addition of cortisol and glucagon) (Wicks, 1968; Mandelli *et al.*, 1973; Rih *et al.*, 1971; Rutter, 1969; Kirby & Hahn, 1973). This induction of tyrosine aminotransferase was, however, also seen in the same explants that exhibited the loss of phenylalanine hydroxylase (Tourian, 1973). The present systematic exploration of several biochemical parameters, including enzymes expected to increase in amount during late gestation, indicates that the behaviour of phenylalanine hydroxylase is not unique. Under the usual culture conditions, which prevent histologically detectable necrosis and permit the induction of tyrosine aminotransferase, the functional integrity of explants is much less satisfactory than one might expect. The various criteria by which one can judge the physiological state of the explants are discussed.

Experimental

Materials

Foetuses (20 days old) were obtained from time-mated albino rats of the Fisher strain. Adult rats were 60–90-day-old males.

Eagle's minimum essential medium with Hanks balanced salts and 1% glutamine, heat-inactivated horse serum and the antibiotic–antimycotic mixture were obtained from Grand Island Biologicals Co., Grand Island, N.Y., U.S.A. Hydrocortisone acetate (to be referred to as cortisol) was obtained from Merck, Sharp and Dohme, West Point, Pa., U.S.A.; crystalline insulin from Sigma Chemical Co., St. Louis, Mo., U.S.A., was solubilized with HCl.

* Present address: Section on Intermediary Metabolism, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

† Present address: Instituto de Investigacion Clinica, Facultad de Medicina, Universidad Del Zulia, Apartado 1151, Maracaibo, Venezuela.

Glucagon from Eli Lilly and Co., Indianapolis, Ind., U.S.A., was dissolved in the diluent provided.

Sterile disposable plastic organ-tissue-culture dishes (60mm×15mm) were supplied by Falcon Plastics, Oxnard, Calif., U.S.A. Metricell filters (13 mm diam.) with a pore size of 0.80 μ m were from Gelman Instruments Co., Ann Arbor, Mich., U.S.A.

Methods

Culture media. The basic culture medium consisted of Eagle's medium (see above), supplemented with NaHCO_3 to give a final concentration of 25mM and with the antibiotic-antimycotic mixture to give 100 units of penicillin, 100 μ g of streptomycin and 0.25 μ g of fungizone per ml of culture medium. Where noted, heat-inactivated horse serum was added to a final concentration of 20% (v/v). Concentrated hormone suspensions were added to obtain final concentrations of 10 μ M-cortisol, 5 μ g of insulin/ml and 10 μ g of glucagon/ml. The pH of the medium was maintained at 7.4 ± 0.1 by gassing continuously with $\text{O}_2 + \text{CO}_2$ (95:5) at 37°C.

Preparation and incubation of explants. Rats were killed by decapitation. Foetuses were removed under sterile conditions and their livers immediately immersed in the sterile culture medium. Livers from six to eight foetuses of the same litter were cut into small cubes and randomized. Ten cubes were placed on each Metricell filter supported by stainless-steel grids. One grid was placed over the central well of the organ culture dish (containing 0.7ml of culture medium) at the air-medium interphase, and incubated at $37 \pm 1^\circ\text{C}$ with a circulating gas phase of $\text{O}_2 + \text{CO}_2$ (95:5) at atmospheric pressure and 100% humidity.

After the indicated periods of culturing the medium was removed and assayed immediately. Explants from two grids (i.e. 20 cubes) were rinsed with 0.15M-KCl and homogenized in 0.5ml of 0.15M-KCl with a motor-driven Teflon-glass homogenizer and centrifuged at 45000g for 60min. The supernatant fluid, referred to below as the 'soluble fraction', was decanted. The pellet was washed and rehomogenized in the same volume of KCl, Triton X-100 was added to a final concentration of 0.5% and after 30min at 0°C it was sonicated (Raytheon Sonic Oscillator) for 3×30s at 0°C; this preparation is referred to below as the 'particulate fraction'.

Analytical procedures. Enzymes were assayed at 25°C under optimum substrate and cofactor concentrations. Previously described methods were used to determine hexokinase (EC 2.7.1.1) (Jamdar & Greengard, 1970), aspartate aminotransferase (EC 2.6.1.1) (Herzfeld & Greengard, 1971), glutamate dehydrogenase (EC 1.4.1.2) (Herzfeld, 1972) and tyrosine aminotransferase (Lin *et al.*, 1958). Malate dehydrogenase (EC 1.1.1.37) was assayed by the method of Ochoa (1955), with twice the concentra-

tions of NADH and of oxaloacetic acid, i.e. 0.1 and 0.5mM respectively. Enzyme activities are expressed as μ mol or nmol of product formed per min.

Protein was determined by the method described by Lowry *et al.* (1951), with bovine serum albumin as the standard. Protein released into the culture medium was precipitated with 9 vol. of cold 5% (w/v) trichloroacetic acid, centrifuged at 5000g for 20min and redissolved in 0.5M-NaOH.

To avoid the inaccuracy involved in weighing small wet tissue fragments the mass of the explants was calculated by comparing the protein content of 20 freshly cut cubes with that of an accurately weighed liver sample obtained from the same foetal litter. After some practice, the size of the cubes became highly reproducible: the mean weight of 20 cubes, 0.046g (i.e. the mean volume of a single cube was about 2.3mm³) varied by less than 10% from experiment to experiment. To express the results per g of liver, the total enzyme activity units or mg of protein in 20 cubes (determined at various times after explantation) were thus divided by 0.046 (i.e. their fresh weight in g at zero time).

Histological examination. Explants were fixed in formalin, dehydrated with ethanol, impregnated first with xylene, then with cedar-wood oil and finally embedded in paraffin. Serial sections of 5 μ m thickness were cut, affixed to glass slides and stained with haematoxylin and eosin.

Results and Discussion

It is known that slices of rat liver, immersed in nutrient media for 1 or 2h, release a considerable portion of their soluble protein content (e.g. Streffer & Williamson, 1965). An estimate of the possible loss of protein from small cubes of foetal rat liver cultured for 1 day or more at a medium-gas interphase has not been included in the several published studies (see the introduction section). The present results show that in such explants, cultured in a serum-free medium ('basic'), the soluble protein concentration decreased from 46.6 to 17.4mg/g in 24h and to 5.8mg/g in 48h. As indicated by the last column of Table 1 much of this protein was recovered in the culture medium. The presence of horse serum in the medium decreased significantly the loss of soluble protein from the explants, but even so, only about 30% of the total was still present in the explants at 48h. The addition of insulin, cortisol and glucagon to the medium, separately or in various combinations, did not exert any significant influence on the protein content of the explants.

It has been suggested that the possible loss of some protein from foetal liver explants may be attributable to the elimination of haematopoietic cells (Wicks, 1968; Kirby & Hahn, 1973). However, according to cytomorphometric analyses (Greengard *et al.*, 1972), haematopoietic tissue accounts for only 38 and 25%

Table 1. *Changes in the protein content of foetal liver explants during culture*

The results are from experiments with ten different foetal litters. Samples from each pool were cultured under several of the conditions listed. Serum, insulin, glucagon and cortisol were added to the basic medium ('basic') at zero time. The means for the 24 h culture with serum include values obtained with as well as without these hormones. —, Not measured. Values refer to pools of livers from 1 litre or to means $\pm$ s.d. of results with numbers of pools in parentheses.

Time of culture (h)	Medium	Protein concentration (mg/g of liver)		
		Particulate	Soluble	Medium
0	'Basic'	61.6 $\pm$ 8.5 (7)	46.6 $\pm$ 4.9 (7)	—
5	'Basic'	—	39.5 $\pm$ 8.3 (4)	4.7 $\pm$ 1.9 (3)
24	'Basic'	—	17.4 $\pm$ 8.4 (6)	18.0 $\pm$ 5.7 (5)
24	Serum ($\pm$ insulin, cortisol, glucagon)	54.7 $\pm$ 7.8 (10)	29.8 $\pm$ 3.1 (10)	—
48	'Basic'	65.0 $\pm$ 13.0 (3)	5.8 $\pm$ 0.8 (6)	19.7 $\pm$ 4.1 (5)
48	Insulin	56.6, 59.0	6.8, 6.6	—
48	Insulin+cortisol+glucagon	49.0, 57.0	8.9, 8.7	—
48	Serum	57.4 $\pm$ 13.5 (6)	13.4 $\pm$ 2.0 (6)	—
48	Serum+insulin+cortisol+glucagon	57.6 $\pm$ 9.1 (6)	16.1 $\pm$ 2.2 (6)	—

Table 2. *Soluble and particulate enzyme activities in cultured foetal liver explants*

All explants (except those denoted by 'basic') were cultured in the presence of serum. Values represent means $\pm$ s.d. (with the numbers of observations in parentheses), which comprise results obtained both without and with the hormones referred to in Table 1 since these were indistinguishable.

Time after explantation (h)	Enzyme activity (μ mol/min per g of foetal liver)		
	Particulate	Soluble	In medium
Malate dehydrogenase			
0		222 $\pm$ 47 (11)	
24		223 $\pm$ 32 (8)	58 $\pm$ 19 (5)
48		96 $\pm$ 23 (8)	173 $\pm$ 21 (5)
48 'basic'		37 $\pm$ 9 (7)	150 $\pm$ 47 (8)
Hexokinase			
0		0.255 $\pm$ 0.076 (5)	
24		0.179 $\pm$ 0.014 (4)	0.0
48		0.052 $\pm$ 0.027 (19)	0.0
Glutamate dehydrogenase			
0	33.9 $\pm$ 6.7 (6)	<1 (2)	
24	22.4 $\pm$ 3.8 (4)		
48	15.7 $\pm$ 2.9 (12)	6.8 $\pm$ 0.5 (3)	2.2 $\pm$ 0.3 (3)
48 'basic'	6.6	13.0	4.0
Aspartate aminotransferase			
0	96 $\pm$ 16 (7)	18.5 $\pm$ 2.3 (5)	
24	44.6 $\pm$ 12.4 (10)	35.8 $\pm$ 4.7 (13)	11.4 $\pm$ 2.7 (5)
48	19.9 $\pm$ 7.8 (18)	23.1 $\pm$ 5.6 (20)	28.5 $\pm$ 4.9 (5)
48 'basic'	7.9 $\pm$ 1.4 (6)	29.1 $\pm$ 4.7 (6)	
Protein (mg/g)			
0	61.6 $\pm$ 8.5 (7)	46.6 $\pm$ 4.9 (7)	
24	54.7 $\pm$ 7.8 (10)	29.7 $\pm$ 3.1 (10)	
48	57.2 $\pm$ 13.5 (6)	12.4 $\pm$ 2.0 (6)	
48 'basic'	65.0 $\pm$ 13.0 (3)	5.8 $\pm$ 0.8 (6)	19.7 $\pm$ 4.1 (5)

of the liver mass at 5 and 2 days before birth respectively, so that even a complete expulsion of these cells would not account for the loss of over 60% of the total soluble protein content observed. Further, there was no decrease in the particulate protein content of

the explants (see Table 1). We thus attribute the loss of soluble protein to leakage from the hepatocytes.

Cortisol and glucagon did not have any effect on the activity of enzymes measured in the experiments

of Table 2, therefore the values obtained with and without these hormones were averaged. The first enzyme, malate dehydrogenase, increases slightly *in vivo* during the last 2 days of gestation, from 192 ± 26 s.d. ($n = 7$) to 234 ± 5 s.d. ($n = 3$) units. After 48 h of culture *in vitro* the total soluble malate dehydrogenase activity (i.e. that retained in the foetal liver explants plus that recovered from the medium) showed a small, statistically insignificant rise, from 222 to 269 units. In the absence of serum ('basic' medium) the total activity was somewhat less and a greater percentage was released into the medium. Comparison with the measurements shown in the last part of Table 2 indicates that this release was proportionate to the release of total soluble protein into the medium. In media containing serum it was not possible to determine the amount of released protein, but it is highly probable that the decrease in soluble protein in the explants themselves, like that of malate dehydrogenase, represents release rather than degradation.

Jamdar & Greengard (1970) showed that the hexokinase activity of foetal liver decreases considerably during the last few days of gestation. Table 2 shows that in explants, too, there was a drastic decrease, but this enzyme, unlike malate dehydrogenase, did not accumulate in the medium.

The glutamate dehydrogenase activity of foetal liver doubles *in vivo* during the last 2 days of gestation (Herzfeld *et al.*, 1973); at no age is there significant activity in the soluble fraction of liver homogenates (Herzfeld, 1972; Herzfeld *et al.*, 1973). In explants, as seen in Table 2, there was some decrease in total glutamate dehydrogenase activity but more strikingly, a considerable portion of the activity was detected in the soluble fraction of the homogenized explants. In the absence of serum this translocation was twice as extensive. A small amount of glutamate dehydrogenase was also detected in the culture medium.

Both the soluble and the mitochondrial variants of aspartate aminotransferase increased in amount during the last 2 days of gestation and both showed additional two- to three-fold rises 1 day after the administration of cortisol *in utero* (Herzfeld & Greengard, 1971). However, in explants cultured in the presence of serum there was a decrease in total aspartate aminotransferase activity, and cortisol was without effect. After the first 24 h of culture there was significant activity in the medium and, as in the case of malate dehydrogenase, about twice as much was released during the second 24 h as during the first. The apparent increase in soluble aspartate aminotransferase during the first 24 h is probably due to the fact that some of the mitochondrial aspartate aminotransferase (like the glutamate dehydrogenase) moved to the soluble cytoplasm within the cells of the explant (cf. Table 2, columns 1 and 2). In the absence of serum the decrease in particulate aspartate aminotransferase was much greater; this accounts for the presence of as much activity in the soluble fraction of these explants as was found in those cultured in the presence of serum.

Wicks (1968) has demonstrated that in foetal liver explants the induction of tyrosine aminotransferase by cortisol and glucagon represents synthesis *de novo*. The activity per mg of soluble protein obtained in the present studies (see Table 3) was similar or higher than that reported by Wicks (1968) and by other laboratories (Räihä *et al.*, 1971; Mandelli *et al.*, 1973; Rutter, 1969; Kirby & Hahn, 1973) using similar culture conditions. Clearly, this considerable increase in specific activity is in part due to a decrease in the denominator, i.e. to the loss of total soluble protein. But even in terms of activity per g the rise (to 25% of that in the adult liver) is remarkable, especially in view of such contraindications of 'health' in the same explant as the release of over 60% of its total soluble protein. It is noteworthy (see Table 3, last column) that tyrosine aminotransferase, unlike other soluble

Table 3. Induction of tyrosine aminotransferase in foetal liver explants

Explants were cultured in basic media supplemented with serum. Cortisol, insulin and glucagon were added at zero time.

	Soluble protein (mg/g)	Tyrosine aminotransferase activity		
		(nmol/min per g of liver)	(nmol/min per mg of soluble protein)	In medium
Adult	95.0	1040	10.6	
Foetus	43.5	Undetectable	Undetectable	
24 h culture				
Serum	26.8	32	1.3	
Serum+cortisol+glucagon	27.7	92	3.7	
Serum+insulin+cortisol+glucagon	29.9	94	3.5	
48 h culture				
Serum	15.1	11	0.8	
Serum+cortisol+glucagon	18.1	310	18.8	Undetectable
Serum+insulin+cortisol+glucagon	17.9	260	15.9	Undetectable

enzymes, was not lost from the explants (a release into the medium of 10% of the total activity would have been measurable by the assay method used). These observations must mean that the functionally intact cells which synthesize tyrosine aminotransferase also retain their total soluble protein content whereas the other cells do not continue their synthetic activities, and these are the ones that release much of their soluble proteins. The existence of two such distinct cell populations in cultures of embryonic chick liver has been postulated by Goodridge *et al.* (1974) to explain the fact that the newly synthesized malate dehydrogenase (decarboxylating) (NADP⁺) (EC 1.1.1.40) was preserved in the cells whereas lactate dehydrogenase (EC 1.1.1.27) was released into the medium.

Histological examination could not identify the functionally deteriorated fraction of the explants. In agreement with several studies of foetal rat liver cultured under similar conditions we saw no signs of necrosis or cell damage in either the inner or outer areas of sections of explants maintained for 48 h in the presence or the absence of serum. As reported for explants of chick embryo liver (Skea & Nemeth, 1969) some tissue disaggregation, seen in the 'basic' medium, was essentially prevented by the presence of serum.

The inadequacy of currently used foetal rat liver culture systems has been obscured by the repeatedly observed, apparent histological normality of the cells, and by the prematurely evoked synthesis of tyrosine aminotransferase, a uniquely responsive, rapidly synthesized enzyme. A system in which only about one-third of the explant cells are capable of continued chemical differentiation is clearly not suitable for studying the mechanism of the spontaneous or induced quantitative (two- to three-fold) increases exhibited by many hepatic enzymes during late foetal differentiation *in vivo*. The failure of such increases to occur *in vitro* could not be ascribed to the absence of physiological factors that operate *in vivo*, but to cellular deterioration of which the release of malate dehydrogenase provides a simple, quantitative measure.

This investigation was supported by U.S. Public Health Service, Grants AM 00567 from the National Institute of Arthritis, Metabolism and Digestive Diseases and CA 08676 from the National Cancer Institute; by Grant RR 05591 from the General Research Support Branch, Division of Research Resources; and by the Fundacion Juan March, Madrid, Spain, and the Program of Cultural Cooperation Between the United States of America and Spain.

References

- Goodridge, A. G., Garay, A. & Silpananta, P. (1974) *J. Biol. Chem.* **249**, 1469–1475
- Greengard, O. (1971) *Essays Biochem.* **7**, 159–205
- Greengard, O. & Dewey, H. K. (1967) *J. Biol. Chem.* **242**, 2986–2991
- Greengard, O., Federman, M. & Knox, W. E. (1972) *J. Cell Biol.* **52**, 261–272
- Herzfeld, A. (1972) *Enzymes* **13**, 246–251
- Herzfeld, A. & Greengard, O. (1971) *Biochim. Biophys. Acta* **237**, 88–98
- Herzfeld, A., Federman, M. & Greengard, O. (1973) *J. Cell Biol.* **57**, 475–483
- Jamdar, S. C. & Greengard, O. (1970) *J. Biol. Chem.* **245**, 2779–2783
- Kirby, L. & Hahn, P. (1973) *Can. J. Biochem.* **51**, 476–481
- Lin, E. C. C., Pitt, B. M., Civen, M. & Knox, W. E. (1958) *J. Biol. Chem.* **233**, 668–673
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mandelli, M., Sereni, L. P. & Sereni, F. (1973) *Biochim. Biophys. Acta* **309**, 44–49
- Ochoa, S. (1955) *Methods Enzymol.* **1**, 735–739
- Räihä, N. C. R., Schwartz, A. L. & Lindross, C. M. (1971) *Pediat. Res.* **5**, 70–76
- Rutter, W. (1969) in *Foetal Autonomy* (Wolstenholme, G. W. E. & O'Connor, M., eds.), pp. 59–78, J. and A. Churchill, London
- Sereni, F. & Sereni, L. P. (1970) *Adv. Enzyme Regul.* **8**, 253–267
- Skea, B. R. & Nemeth, A. M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 795–802
- Streffer, C. & Williamson, D. H. (1965) *Biochem. J.* **95**, 552–560
- Tourian, A. (1973) *Biochim. Biophys. Acta* **309**, 44–49
- Wicks, W. D. (1968) *J. Biol. Chem.* **243**, 900–906
- Yeung, D. & Oliver, I. T. (1968) *Biochem. J.* **108**, 325–331