Insulin-responsive cultured foetal-rat hepatocytes

Their preparation and characterization

Diane C. DeSANTe,* Leah LITTLE,* Daniel E. PEAVY*† and Frank VINICOR*†§

*Veterans Administration Medical Center and Departments of †Medicine and †Physiology, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A.

(Received 17 February 1984/Accepted 13 June 1984)

An improved non-perfusion method for the preparation of cultured foetal-rat hepatocytes is described. Digestion of the liver with collagenase and deoxyribonuclease I gave yields of 40×10^6 hepatocytes/g of liver. The plating efficiency of hepatocytes in medium with $10 \mu\text{M}$ -cortisol was 50%. Cell morphology and metabolism were maintained through 3 days of monolayer culture, with minimal contamination by haematopoietic cells or fibroblasts. The cultured cells bound and degraded ^{125}I -insulin in a time- and dose-dependent manner. The estimated ED_{50} for competitive binding at 37°C was 1.1 nM. Curvilinear Scatchard plots were observed, with estimates of 16 500 high-affinity sites ($K_d = 813 \text{ pM}$) and 53 000 low-affinity sites ($K_d = 23 \text{ nM}$) per cell. The cultured cells demonstrated a glycogenic response to insulin, with an estimated ED_{50} of 120 pM. The degree of glycogenic response to insulin varied with time in culture: 500% above basal on day 1, 200% on day 2, and only 150% on day 3. Cultured foetal cells also exhibited a time-dependent uptake of 2-aminoisobutyric acid, which, in contrast with previous reports with adult cells, was not stimulated by the presence of 10 nM-insulin. Cultured foetal hepatocytes may provide an interesting model with which to study the relationship between insulin-receptor binding and insulin action.

Several investigations have documented an anabolic action of insulin on a variety of foetal metabolic and growth processes during late gestation (Fricke & Clark, 1973; Glinsmann *et al.*, 1975; McCormick *et al.*, 1979; Bourbon & Gilbert, 1981). This effect of insulin is important both for ensuring growth during the foetal period and for maintenance of euglycaemia during the first few hours of neonatal life (Girard *et al.*, 1973; Vinicor *et al.*, 1976; Hay, 1979).

In studies of the mechanisms of insulin action during this perinatal period, specific insulin receptors have been identified in foetal tissues which by functional criteria are similar to insulin receptors in adult cells (Kelly *et al.*, 1974; Neufeld *et al.*, 1980; Menuelle & Plas, 1981; Vinicor & Kiedrowski, 1982; Plas & Desbuquois, 1982). Except for the studies by Plas and associates, these

studies have been performed with isolated hepatic membranes (Kelly *et al.*, 1974; Neufeld *et al.*, 1980; Vinicor & Kiedrowski, 1982; Sara *et al.*, 1983) or cell preparations (Blazquez *et al.*, 1976; Sodoyez-Goffaux *et al.*, 1980; Autuori *et al.*, 1981). Interpretation of the results of these studies is limited by the possible inclusion of membrane particles or cells of non-parenchymal origin. Furthermore, when isolated membranes are utilized, the investigation of the relationship between insulin binding and intracellular biochemical consequences of insulin action, such as increased glycogenesis or protein synthesis, is precluded.

To investigate further the ontogenesis of insulin-hepatocyte interaction in the developing rat liver, including the relationship between insulin processing and intracellular biochemical events, we have developed a technique to prepare isolated foetal hepatocytes in primary culture in high yield and purity which are metabolically active and hormonally responsive.

Preliminary results of this investigation have been presented at a meeting of the Midwest

§ To whom reprint requests should be addressed at: Endocrinology Section (111E), Veterans Administration Medical Center, 1481 W. 10th Street, Indianapolis, IN 46202, U.S.A.

Section of the American Federation for Clinical Research, Chicago, November 2–5, 1983 (DeSante *et al.*, 1983).

Materials and methods

Materials

Rats were obtained from Harlan Industries, Indianapolis, IN, U.S.A. Earle's Balanced Salt Solution (Earle, 1943) without NaHCO_3 , Leibovitz L-15 media (Leibovitz, 1963) and foetal bovine serum were from Grand Island Biological Co. (GIBCO), Grand Island, NY, U.S.A. Collagenase was from Worthington Diagnostics, Freehold, NJ, U.S.A., and deoxyribonuclease I was from Boehringer/Mannheim Biochemicals, Indianapolis, IN, U.S.A. Bovine dermal collagen (Vitrogen 100TM) was from Flow Laboratories, McLean, VA, U.S.A. Bovine serum albumin (fraction V) was from Sigma Chemical Co., St. Louis, MO, U.S.A., or GIBCO; reagent-grade albumin for glycogenesis studies was from Pentex, Miles Laboratories, Elkhart, IN, U.S.A. Cortisol was from Merck, Sharpe and Dohme, West Point, PA, U.S.A. Other chemicals were obtained from standard commercial sources. Pig insulins (mono[¹²⁵I]iodo and native) were generously given by Dr. B. Frank, Eli Lilly and Co., Indianapolis, IN, U.S.A. [¹⁴C]-Uridine and Aquasol-2 were from New England Nuclear, Boston, MA, U.S.A. D-[¹⁴C]Glucose and 2-amino[1-¹⁴C]isobutyric acid were from Amersham Corp., Arlington Heights, IL, U.S.A. Tissue-culture flasks (Falcon 3013) and Nalgene membrane filters were from VWR Scientific, Chicago, IL, U.S.A.

Hepatocyte isolation and culture

Timed-pregnant (19 or 20 days) Sprague-Dawley rats were decapitated and the uteri obtained aseptically through an abdominal incision. Foetuses were sterily removed in a laminar-flow hood and decapitated. The livers were removed and placed in cold (15°C) Ca^{2+} - and Mg^{2+} -free Earle's Balanced Salt Solution containing 25 mM-Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid], pH 7.4 (EH buffer). Sterile techniques were used throughout the procedure, including filtration of all solutions through 0.2 μm -pore-size Nalgene filters. After being weighed, the livers (about 0.2 g each) were rinsed twice, cleaned of extraneous tissues and minced with a pair of scalpels to pieces about 3 mm to a side. The EH buffer was aspirated and the liver tissue rinsed three times with 5 vol. (v/w) of the same buffer containing 0.5 mM-EGTA. The livers were then preincubated for 5 min at 37°C in the last EH-buffer rinse. EGTA was removed by three rinses with EH buffer.

The liver tissue was then digested with 5 vol. (v/w) of a solution that contained 250 μg of collagenase [type II; 240 units/mg (1 unit of collagenase liberates 1 μmol of L-leucine equivalents from collagen in 5 h at 37°C and pH 7.5)]/ml of EH buffer plus 1 mM- MgSO_4 , 2.5 mM- CaCl_2 , 2% (v/v) foetal bovine serum and 0.7–1.0 μg of deoxyribonuclease I (approx. 1000 Kunitz units/mg)/ml. In general, the tissue was digested with three successive 15 min incubations in a 37°C Dubnoff shaking water bath. After each incubation, the supernatant containing isolated cells was harvested and the remaining tissue subjected to further digestion. Specifically, after each incubation the digest was diluted with an equal volume of EH + 3 medium (EH buffer plus 1.8 mM- CaCl_2 , 0.81 mM- MgSO_4 and 1% albumin) and allowed to settle briefly. The supernatant was aspirated and filtered through a 100 μm -pore-size nylon mesh. Residual undigested tissue was then lightly triturated five times with a pipette in 10–15 ml of EH + 3 medium, allowed to settle, and the supernatant filtered and combined with the first filtrate. The filter was washed with 5–10 ml of EH + 3 and this filtrate was added to the combined filtrates. The residual tissue was then subjected to two additional collagenase digestions.

The filtered cells were pelleted by centrifugation for 2 min at 50g at 5°C. The supernatant was discarded and the cells were resuspended in 20 ml of Leibovitz L-15 culture medium plus 10% foetal bovine serum. Hepatocytes were allowed to sediment under gravity on ice for 20 min. Each subsequent cell pellet from the collagenase digest was combined with the previous ones and allowed to sediment again.

The final pellet was resuspended in 20 vol. of L-15 medium; small samples were removed for cell counting and determination of viability by Trypan Blue exclusion. Depending on yield, 2×10^6 – 3×10^6 cells were plated per flask in L-15 medium plus 10 μM -cortisol on collagen-coated 25 cm² tissue-culture flasks and incubated at 37°C. Cells were allowed to attach overnight, and non-attached cells (non-viable hepatocytes and haematopoietic cells) and debris were removed by aspiration and filtration of the media through a 0.2 μm Nalgene filter. The cells were then washed twice with 2 ml of L-15 medium/flask, and incubation continued in the filtered 'conditioned' medium plus 10% (v/v) fresh L-15 medium with 100 μM -cortisol (final cortisol concn. 10 μM).

Assay techniques

Cells were washed and then counted microscopically (magnification $\times 500$) in six fields of vision/flask. To estimate the total number of cells per flask, the average count/field in duplicate or

triplicate flasks was divided by the fractional area of the field, which was calibrated with a haemocytometer grid.

DNA content/flask was determined by the fluorimetric method of Cesarone *et al.* (1979), with highly polymerized calf thymus DNA as the standard.

Protein content was determined by a modification of the micro-biuret method of Goa (1953). Bovine serum albumin (fraction V; GIBCO) was used as the standard.

Insulin-binding and -degradation studies

The cultured cells were washed with L-15 medium and then incubated at 37°C with tracer with or without 2 µM unlabelled insulin in a final volume of 1.5 ml of L-15 medium + 1% albumin. The tracer was mono[¹²⁵I]iodinated pig insulin, specifically labelled at either the A-14 or B-26 tyrosine residue to a specific radioactivity of 320–340 µCi/µg, and was used at a final concentration of 40 pM. Non-specific binding or degradation, determined in the presence of 2 µM unlabelled pig insulin, was subtracted from insulin bound or degraded in the presence of tracer only. Non-specific binding for the Scatchard (1949) analysis was treated as an unknown and was determined by the LIGAND program (Munson & Rodbard, 1980).

Incubations were terminated by quickly aspirating the media into tubes containing 1.6 µmol of bacitracin and washing the cell monolayer with 5 × 2 ml of ice-cold EH + 3 medium, all within 1 min. The flasks were placed on ice and the cells scraped and transferred in three consecutive 0.7 ml washes of 10% (w/v) trichloroacetic acid to tubes containing 1 mg of albumin in 10 µl. Samples of the aspirated media were also precipitated with 10% trichloroacetic acid. After centrifugation (10 min, 500g, 5°C), the resulting pellets and supernatants were separated and counted for radioactivity in a Tracor Analytic model 1285 gamma counter with a counting efficiency of 85%.

Glycogenesis studies

The incorporation of [¹⁴C]glucose into glycogen was measured by a modification of the method of Van Handel (1965). The cultured cells were washed two or three times with EH + 3 medium containing 1% albumin (Pentex), 11 mM-D-glucose and 10 µM-cortisol. Incubation in the same medium was initiated by the addition of [¹⁴C]glucose (final sp. radioactivity 0.4 µCi/mg) and various amounts of unlabelled pig insulin. After 150 min at 37°C, the cells were washed three times with cold EH + 3 medium, and the glycogen fraction was isolated by KOH extraction and ethanol precipitation (Van Handel, 1965). Radioactivity in a sample of the

final extract was determined by liquid-scintillation counting (counting efficiency 75%).

Amino acid-transport studies

Cultured cells were washed twice in 2 ml of EH + 3 medium and preincubated for 2 h at 37°C in 1.5 ml of EH + 3 medium with or without 10 nM-insulin. The incubation period was initiated with the addition of 2-amino[¹⁴C]isobutyric acid. At the appropriate times, cells were harvested for determination of trichloroacetic acid-soluble radioactivity in a manner similar to that described above for the insulin-binding studies. Extracellular water in the cell pellet was estimated with [³H]inulin.

Results

Cell preparation and culture

Cell yields were routinely 30×10^6 – 50×10^6 viable hepatocytes/g wet wt. of liver, with the optimum yield being 40×10^6 . Lesser yields were usually accompanied by clumping of cells, indicating inadequate digestion or a deoxyribonuclease I preparation of lower activity. Higher-yield preparations, although accompanied by 90–95% viability as determined by Trypan Blue exclusion, were nevertheless characterized by lower percentage recoveries of attached cells on day 1 of culture. The ratio of collagenase to wet liver weight was important for good yields and needed to be determined for each batch of collagenase.

Viabilities for the hepatocyte preparation (determined with Trypan Blue) were routinely at least 86–92%. Hepatocytes represented about 35–48% of the total cell number, which compares with 34% in whole liver at this stage of development (Greengard *et al.*, 1972). Recovery of attached hepatocytes on day 1 of culture was routinely 50–60% of the 'Trypan-Blue viable' hepatocytes plated on day 0. The number of cells attached on day 1 did not change significantly during 3 days of culture, but decreased after that. Haematopoietic cells were approx. 7% of the total cell number in the flask after washing twice on day 1, and decreased with additional washing.

The DNA content averaged $33.5 \pm 1.0 \mu\text{g}/10^6$ hepatocytes (mean $\pm$ S.E.M.) on day 1 and did not change significantly through 3 days of culture. Occasionally, slightly higher values were observed on day 3 because of a small number of fibroblasts (3% of total cell number).

Protein assays indicated a protein concentration of $217 \pm 39 \mu\text{g}$ (mean $\pm$ S.E.M.) of protein/ 10^6 hepatocytes. These numbers are in agreement with other published reports (Plas & Nunez, 1976).

Electron microscopy indicated normal mononucleated cells with typical hepatocyte morphology.

Insulin binding and degradation

Cultured foetal-rat hepatocytes both bound and degraded ^{125}I -insulin in a time-dependent fashion (Fig. 1). The maximum amount of 'cell-associated' ^{125}I -insulin was 2.5% of tracer at 40 pM (approx. 1 fmol/ 10^6 cells), and was observed at 45 min at

Fig. 1. Time course of ^{125}I -insulin binding and degradation by cultured foetal-rat hepatocytes

Hepatocytes (1.4×10^6 cells/flask) in culture for 2 days were incubated with 40 pM ^{125}I -insulin with or without 2 μM unlabelled insulin in L-15 medium + 1% albumin. At the indicated times, samples of the media and the washed cell pellet were assayed for trichloroacetic acid precipitability. (a) The 'cell-associated' label represents the sum of the trichloroacetic acid-soluble and -insoluble fractions associated with the cell pellet after washing of the monolayer five times with ice-cold EH+3 buffer. Results are expressed as percentages of the total tracer added to each flask after subtraction of the non-specific binding determined in the presence of 2 μM unlabelled insulin. (b) The points represent the fraction of the cell-associated radioactivity that is trichloroacetic acid-soluble at the indicated times. (c) The points represent the fraction of the radioactivity present in the extracellular media that is trichloroacetic acid-soluble at the indicated times.

37°C (Fig. 1a). (We prefer to use the term 'cell-associated' insulin rather than the more prevalent term insulin 'binding', because the former includes internalized and degraded insulin, whereas the latter implies only that portion of insulin that is receptor-bound and not internalized.) Of the tracer that was cell-associated in this experiment, 9% was degraded at 45 min, a percentage that remained constant up to 2 h of incubation (Fig. 1b). Degraded ^{125}I -insulin in the extracellular medium (Fig. 1c) accumulated in a nearly linear fashion over 2 h after a lag time of 20–30 min (Fig. 1c). Under these assay conditions, cells do not release insulin-degrading activity into the media (results not shown); thus these results suggest that degraded ^{125}I -insulin appearing in the media must first have been in contact with the cultured foetal hepatocytes.

Results of competitive binding studies of ^{125}I -insulin under maximum binding conditions (45 min at 37°C) are shown in Fig. 2. Under these conditions the total degradation of tracer was less than 6% of the total ^{125}I -insulin added (40 pM). Increasing concentrations of unlabelled insulin displaced ^{125}I -insulin in a dose-dependent fashion, with half-maximal displacement (ED_{50}) occurring

Fig. 2. Competitive binding of ^{125}I -insulin and unlabelled insulin by cultured foetal-rat hepatocytes

Cells in culture for 1 day were incubated for 45 min at 37°C with 40 pM ^{125}I -insulin and various concentrations of unlabelled insulin in L-15 medium + 1% albumin. The cells were then washed five times with cold EH+3 buffer and scraped in 10% (w/v) trichloroacetic acid. The radioactivity in the cell pellet was determined and is expressed as a percentage of that bound in the presence of tracer only. The points are the means $\pm$ S.E.M. for two experiments, each performed in triplicate. These data were subjected to Scatchard analysis (inset): bound (B) and free (F) insulin were expressed in pmol/l of incubation mixture.

at 1.1 nM when the data were fitted by the PREFIT and ALLFIT programs of DeLean *et al.* (1978). Curvilinear Scatchard plots were observed in these cultured foetal hepatocytes (Fig. 2, inset), suggesting either different classes of insulin receptors or site-site interactions among receptors (Feldman, 1972; De Meyts *et al.*, 1973). The LIGAND-fitted (Munson & Rodbard, 1980) curve yielded estimates of 16 500 binding sites/cell with an affinity constant (K_d) of 813 pM and 53 000 binding sites with an affinity constant of 23.3 nM. A significantly ($P < 0.001$) improved fit was obtained when the two-site model rather than the one-site model was used.

Glycogenesis studies

As a measure of the functional integrity of these cultured foetal hepatocytes, the incorporation of

Fig. 3. Effect of insulin on incorporation of [¹⁴C]glucose into glycogen

Foetal hepatocytes in culture for 1 day were incubated with 11 mM-[¹⁴C]glucose (0.4 μCi/mg), 10 μM-cortisol and the indicated insulin concentration in 1.5 ml of EH + 3 buffer for 2.5 h at 37°C. The incubation was terminated by washing the cells three times with ice-cold EH + 3 buffer. Cells were disrupted and collected by scraping with three successive 0.6 ml portions of 30% (w/v) KOH. The scrapings were added to tubes containing 4 mg of rabbit liver glycogen as carrier and incubated for 20 min in a boiling-water bath. After chilling, 2 vol. of 95% (v/v) ethanol and 25 μl of saturated Na₂SO₄ were added. The tubes were chilled in an ice bath for 20 min and then centrifuged (20 min, 1250 g, 5°C). The pellets were drained and resuspended in 1.0 ml of water and re-precipitated with 2.0 ml of 95% ethanol. After centrifugation as above, the pellets were dissolved in 1.0 ml of 0.01 M-HCl. Portions (0.9 ml) were counted for radioactivity in 9.0 ml of Aquasol-2.

[¹⁴C]glucose into glycogen in the presence of various insulin concentrations was determined. Insulin exhibited a dose-dependent effect on glucose incorporation by these cells (Fig. 3). Estimated half-maximal and maximal stimulations of [¹⁴C]glucose incorporation into glycogen were observed at insulin concentrations of 120 pM and 10 nM respectively.

The effect of insulin on glycogenesis varied with time in culture (Table 1). Basal [¹⁴C]glucose incorporation by 19-day-foetal cells in the absence of insulin averaged 533 c.p.m./2.5 h per 10⁶ cells on day 1, 1428 on day 2 and 670 on day 3. Maximal stimulation was 400–600% of control at 10 nM-insulin on day 1 of culture and declined to 150% by day 3. Thus, although insulin enhanced glycogen synthesis regardless of time in culture, the absolute and relative (i.e. percentage stimulation over basal) effects of insulin varied with the number of days of culture.

Amino acid-transport studies

Uptake of 2-aminoisobutyric acid was time-dependent, and approached a steady-state value of 12 nmol/10⁶ cells in 2 h (Fig. 4). In the presence of 10 nM-insulin, uptake appeared to be slightly stimulated at 2 h, but not statistically significantly. This pattern was maintained for 3 days of culture (results not shown). Although these results contrasted with the commonly observed insulin stimulation of transport system A in adult hepatocytes (for reviews see Guidotti *et al.*, 1978; Kilberg, 1982), they were in agreement with other results with perinatal hepatocytes (Handlogten & Kilberg, 1982).

Table 1. Effect of insulin on glycogenesis in foetal hepatocytes cultured for different periods of time

The effect of insulin on the rate of [¹⁴C]glucose incorporation into glycogen was determined in cells cultured for the indicated number of days. Details of the incubation conditions and the glycogen extraction are presented in the Materials and methods section and in the legend to Fig. 3. Results shown are means ± S.E.M. for *n* experiments: ^asignificantly different from control (basal), $P < 0.01$; ^bsignificantly different from control (basal), $P < 0.05$. Statistical comparisons were made by a paired *t*-test.

Time in culture (days)	n	[¹⁴ C]Glucose incorporated (c.p.m./10 ⁶ cells)	
		Basal	+ 10 nM-Insulin
1	4	533 ± 54	2596 ± 417 ^a
2	3	1428 ± 406	2915 ± 480 ^a
3	3	670 ± 97	1022 ± 199 ^b

Fig. 4. Time course of amino[^{14}C]isobutyric acid uptake with or without 10nM-insulin

Foetal hepatocytes in culture for 2 days were preincubated for 2 h at 37°C in 1.5 ml of EH+3 buffer with (●) or without (○) 10nM-insulin. At zero time, 0.5 mM-amino[^{14}C]isobutyric acid was added to the flasks (0.1 $\mu\text{Ci/ml}$ final concn.) and the incubation was continued at 37°C. At the indicated times the reaction was terminated by quickly aspirating the incubation media and washing the cell monolayer with 4 $\times$ 2 ml of ice-cold EH+3 buffer. Each flask was scraped with 3 $\times$ 0.7 ml of 10% (w/v) trichloroacetic acid. After centrifugation (10 min, 500g, 5°C), samples of the supernatants were counted for radioactivity in Aquasol 2 (counting efficiency 70%). Results shown are means $\pm$ S.E.M. for six flasks from two experiments, each normalized to the same cell count/flask (1.4×10^6 /flask).

Uridine-incorporation studies

Cultured foetal hepatocytes exhibited a time-dependent incorporation of [^{14}C]uridine into trichloroacetic acid-insoluble material throughout 3 days of culture (results not shown), indicating the sustained viability of these cells (Baur *et al.*, 1975).

Discussion

The development of the foetal liver into the adult organ remains an interesting and important question. Of particular interest is the maturation of the hepatic response to insulin. Several reports have suggested that the foetal liver differs from adult liver both in biological responses to the hormone and in the processing and metabolism of the

insulin-receptor complexes. For example, the foetal liver has been reported not to down-regulate the insulin receptor, in spite of high concentrations of circulating insulin (Neufeld *et al.*, 1980; Vinicor & Kiedrowski, 1982). Although reports of inactive but immunoreactive circulating insulin in the foetus (Felix *et al.*, 1975; Watts & Gain, 1984) provide a possible explanation for this *in vivo*, cultured foetal hepatocytes also appear not to down-regulate appropriately in response to high concentrations of insulin (Menuelle & Plas, 1981). In addition to alterations in receptor processing, differences in the post-receptor metabolism of insulin have also been reported in foetal hepatocytes. *N*-Ethylmaleimide, a potent inhibitor of insulin-degrading enzymes and of insulin degradation by intact adult cells, has been reported not to inhibit insulin degradation by foetal hepatocytes (Plas & Desbuquois, 1982). The post-receptor processing of insulin is of considerable interest because of suggestions that interference with this process may alter cellular responses to insulin. In the adult hepatocyte, inhibition of insulin processing by dansylcadaverine blocks the insulin stimulation of alanine transport and the inhibition of intracellular protein degradation, but not the stimulation of glycogen synthesis (Draznin & Trowbridge, 1982; Peavy *et al.*, 1984). Chloroquine has no effect on insulin action. In the foetal cell, chloroquine has been reported to block insulin-stimulated glycogen synthesis (Plas & Desbuquois, 1982). Thus there are important differences between foetal and adult cells. An understanding of the intracellular events could lead to an increased understanding of post-receptor events in insulin action.

To explore this in depth, a viable, hormonally responsive, foetal preparation which is not contaminated with non-hepatic cells is required. The present paper describes such a system and demonstrates that these cells both bind and respond to insulin. The present method is a significant refinement of published techniques for preparing foetal hepatocytes (Plas, 1969; Leffert & Paul, 1972; Plas *et al.*, 1973; Bellemann *et al.*, 1977). In agreement with Conti Devirgiliis *et al.* (1981), the use of a mixture of crude collagenase and deoxyribonuclease I as the digesting medium resulted in high yields of isolated cells and substantially improved recoveries [55%, compared with 10% reported by Leffert & Paul (1972) and Plas *et al.* (1973)] of attached, viable, functional cells in culture. Furthermore, the ability to culture sterily without antibiotics was a significant achievement, and will be especially useful for studies where antibiotics are known to interfere with peptide-hormone metabolism, such as studies of insulin degradation (Plas & Desbuquois, 1982).

Cortisol ($10\mu\text{M}$) has been used routinely as a supplement to our serum-free media because of the well-documented value of corticosteroids in (1) promoting cell attachment and survival (Laishes & Williams, 1976b; Williams *et al.*, 1978), (2) retention of liver epithelial-cell morphology (Murison, 1976; Marceau *et al.*, 1980; Laishes & Williams, 1976b), (3) inhibition of fibroblast growth (Noyes, 1973; Murison, 1976; Marceau *et al.*, 1980), (4) promotion of glycogen storage and insulin-stimulated glycogen synthesis (Plas *et al.*, 1973; Plas & Nunez, 1976) and (5) stimulation of insulin-receptor synthesis (Salhanick *et al.*, 1983). Although systematic studies of reported cortisol effects (Laishes & Williams, 1976b; Marceau *et al.*, 1980) have not been performed in our culture system, we have observed (1) significant increases in plated-cell recoveries in the presence of $10\mu\text{M}$ -cortisol (55% recovery, compared with 30% without cortisol), (2) inhibition of fibroblast growth, and (3) a glycogenic response to insulin. Systematic studies on other possible growth factors, such as transferrin (Marceau *et al.*, 1982) or insulin (Laishes & Williams, 1976a; Armato *et al.*, 1978), have not been carried out as yet.

The demonstration that foetal cells in culture perform such complex metabolic functions as uptake and degradation of insulin, glycogenesis, amino acid transport and RNA synthesis, attests to their integrity and sustained viability. Characteristics of insulin-receptor binding, including curvilinear Scatchard plots, were similar to those reported previously in foetal-liver plasma membranes (Neufeld *et al.*, 1980; Vinicor & Kiedrowski, 1982; Sara *et al.*, 1983) and in adult hepatocytes (Feldman, 1972; De Meyts *et al.*, 1973; Freychet *et al.*, 1974). Concentrations of insulin which elicited a glycogenic response were in the range of physiological insulin concentrations in portal blood (Blackard & Nelson, 1970) and are in good agreement with published reports on insulin's glycogenic effect in foetal hepatocytes (Menuelle & Plas, 1981). The absence of an effect of insulin on amino acid transport is also in agreement with previously published results (Handlogten & Kilberg, 1982). The contrasting effects of insulin on glycogenesis and amino acid transport may represent differing rates of maturation of these hormone-sensitive systems, or may represent differing degrees of maturation of mechanisms specifically involved in insulin action. Further investigation of insulin binding, processing and action in cultured foetal hepatocytes should provide valuable insights into these processes as they occur in adult liver cells.

We acknowledge the preliminary work of Dr. Leah Kiedrowski and the many helpful discussions and contributions of Dr. William C. Duckworth. This work

was supported by Veterans Administration Research Funds.

References

- Armato, U., Draghi, E. & Andreis, P. G. (1978) *Endocrinology (Baltimore)* **102**, 1155-1166
- Autuori, F., Baldini, P., Ciofi Luzzato, A., Conti Devirgiliis, L., Dini, L., Incerpi, S. & Luly, P. (1981) *Biochim. Biophys. Acta* **678**, 1-6
- Baur, H., Kasperek, S. & Pfaff, E. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 827-838
- Bellemann, P., Gebhardt, R. & Mecke, D. (1977) *Anal. Biochem.* **81**, 408-415
- Blackard, W. G. & Nelson, N. C. (1970) *Diabetes* **19**, 302-306
- Blazquez, E., Rubalcava, B., Montesano, R., Orci, L. & Unger, R. H. (1976) *Endocrinology (Baltimore)* **98**, 1014-1023
- Bourbon, J. & Gilbert, M. (1981) *Biol. Neonate* **40**, 38-45
- Cesarone, C. F., Bolognesi, C. & Santi, L. (1979) *Anal. Biochem.* **100**, 188-197
- Conti Devirgiliis, L., Dini, L., DiPierro, A., Leoni, S., Spagnuolo, S. & Stefanini, S. (1981) *Cell. Mol. Biol.* **27**, 687-694
- DeLean, A., Munson, P. J. & Rodbard, D. (1978) *Am. J. Physiol.* **235**, E97-E102
- De Meyts, P., Roth, J., Neville, D. M., Jr., Gavin, J. R., III & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun.* **55**, 154-161
- DeSante, D. C., Peavy, D. E., Duckworth, W. C. & Vinicor, F. (1983) *Clin. Res.* **31**, 738A
- Draznin, B. & Trowbridge, M. (1982) *J. Biol. Chem.* **257**, 11988-11993
- Earle, W. R. (1943) *J. Natl. Cancer Inst.* **4**, 165-212
- Feldman, H. A. (1972) *Anal. Biochem.* **48**, 317-338
- Felix, J. M., Sutter-Dub, M. T. & Legrele, C. (1975) *Horm. Metab. Res.* **7**, 394-399
- Freychet, P., Rosselin, G., Rancon, F., Fouchereau, M. & Broer, Y. (1974) *Horm. Metab. Res. Suppl. Ser.* **5**, 72-78
- Fricke, R. & Clark, C. M., Jr. (1973) *Am. J. Physiol.* **224**, 117-121
- Girard, J., Cuendet, G., Marlis, E., Kervan, A., Rieutort, M. & Assan, R. (1973) *J. Clin. Invest.* **52**, 3190-3200
- Glinemann, W. H., Eisen, H. J., Lynch, A. & Chez, R. A. (1975) *Pediatr. Res.* **9**, 600-604
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218-222
- Greengard, O., Federman, M. & Knox, W. E. (1972) *J. Cell Biol.* **52**, 261-272
- Guidotti, G. G., Borghetti, A. F. & Gazzola, G. C. (1978) *Biochim. Biophys. Acta* **515**, 329-366
- Handlogten, M. E. & Kilberg, M. S. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1113-1119
- Hay, W. (1979) *Semin. Perinatol.* **3**, 157-176
- Kelly, P. A., Posner, B. I., Tsushima, T. & Friesen, H. G. (1974) *Endocrinology (Baltimore)* **95**, 532-539
- Kilberg, M. S. (1982) *J. Membr. Biol.* **69**, 1-12
- Laishes, B. A. & Williams, G. M. (1976a) *In Vitro* **12**, 521-532
- Laishes, B. A. & Williams, G. M. (1976b) *In Vitro* **12**, 821-832

- Leffert, H. L. & Paul, D. (1972) *J. Cell Biol.* **52**, 559–568
- Leibovitz, A. (1963) *Am. J. Hyg.* **78**, 173–180
- Marceau, N., Goyette, R., Valet, J. P. & Deschenes, J. (1980) *Exp. Cell Res.* **125**, 497–502
- Marceau, N., Noel, M. & Deschenes, J. (1982) *In Vitro* **18**, 1–11
- McCormick, K. L., Susa, J. B., Widness, J. A., Singer, D. B., Adamsons, K. & Swartz, R. (1979) *Diabetes* **28**, 1964–1068
- Menuelle, P. & Plas, C. (1981) *Diabetologia* **20**, 647–653
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Murison, G. L. (1976) *Exp. Cell Res.* **100**, 439–443
- Neufeld, N. D., Scott, M. & Kaplan, S. A. (1980) *Dev. Biol.* **78**, 151–160
- Noyes, W. F. (1973) *Proc. Soc. Exp. Biol. Med.* **144**, 245–248
- Peavy, D. E., Edmondson, J. W. & Duckworth, W. C. (1984) *Endocrinology (Baltimore)* **114**, 753–760
- Plas, C. (1969) *C. R. Hebd. Seances Acad. Sci. Ser. D* **268**, 143–146
- Plas, C. & Desbuquois, B. (1982) *Biochem. J.* **202**, 333–341
- Plas, C. & Nunez, J. (1976) *J. Biol. Chem.* **251**, 1431–1437
- Plas, C., Chapeville, F. & Jacquot, R. (1973) *Dev. Biol.* **32**, 82–91
- Salhanick, A. I., Krupp, M. N. & Amatruda, J. M. (1983) *J. Biol. Chem.* **258**, 14130–14135
- Sara, V. R., Hall, K., Misaki, M., Fryklund, L., Christensen, N. & Welterberg, L. (1983) *J. Clin. Invest.* **71**, 1084–1094
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Sodoyez-Goffaux, F., Sodoyez, J., Thiry-Moris, W. & DeVos, C. (1980) *Diabetes* **29**, 97a
- Van Handel, E. (1965) *Anal. Biochem.* **11**, 256–265
- Vinicor, F. & Kiedrowski, L. (1982) *Endocrinology (Baltimore)* **110**, 782–790
- Vinicor, F., Higdon, G., Clark, J. & Clark, C. M. (1976) *J. Clin. Invest.* **58**, 571–578
- Watts, C. & Gain, K. (1984) *Diabetes* **33**, 50–56
- Williams, G. M., Bermudez, E., San, R. C. H., Goldblatt, P. J. & Laspia, M. F. (1978) *In Vitro* **14**, 824–837