Immunoreactive insulin from mouse brain cells in culture and whole rat brain

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Foetal mouse brain cells were cultured as described previously [Sotelo, Gibbs, Gajdusek, Toh & Wurth (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 653–657] without added insulin and without foetal calf serum after 12 days in culture. Examination by phase-contrast microscopy showed that these modifications did not appear to affect growth and development of the cells adversely. Silver impregnation of the cultures and indirect immunofluorescence following reaction with tetanus toxin showed that a high proportion of the cells resembled neurones. Analysis of concentrated culture medium by radioimmunoassay and high-pressure liquid chromatography (h.p.l.c.) revealed that the cells produced two main forms of immunoreactive insulin which differed from authentic pancreatic insulin in retention time. Immunoreactive somatostatin was also produced in culture and this was resolved into at least three forms by h.p.l.c. Immunoreactive insulin was also extracted from whole rat brain by using two published procedures. The method of Havrankova, Schmechel, Roth & Brownstein [*Proc. Natl. Acad. Sci. U.S.A.* (1978) **75**, 5737–5741] consistently gave greater yields of insulin than did that of Eng & Yalow [*Diabetes* (1980) **29**, 105–109] and the concentration was about three times that of plasma. The extracted insulin was further characterized by h.p.l.c. in each case and was found to behave like authentic pancreatic insulin. The production of insulin and somatostatin by foetal mouse brain cells in culture suggests that they may be a useful model system for studies of neuropeptide biosynthesis.

Preparatory to the investigation of steroid transformations in rodent brain we established cultures of central neurones from foetal mouse brain by the method of Sotelo *et al.* (1980) and noted that the addition of insulin to the medium was not required for the initiation and maintenance of cell growth. This observation prompted the question of whether brain cells in culture synthesize their own insulin requirement, in view of the findings of Sotelo *et al.* (1980) who believed that the inclusion of large amounts of glucose and insulin in the medium was essential for the maturation and longterm survival of cultured neurones.

Earlier reports of insulin in mammalian brain were at odds. Havrankova *et al.* (1978) stated that insulin concentrations in acid-ethanol extracts of

brain were 25 times those found in plasma. Similar results were obtained in the same laboratory by Rosenzweig *et al.* (1980) who also gave concentrations of pig insulin-like material in several guinea pig tissues, including brain. In contrast, Eng & Yalow (1980, 1981) reported that the insulin content of rat, rabbit and dog brain was generally less than that found in plasma, and Eng & Yalow (1982) concluded that 'it is unlikely that non-pancreatic guinea pig tissues contain or synthesize a peptide resembling pork or other non-guinea pig mammalian insulin'.

In view of these disparate accounts it was decided to extract rat brain using the procedures of Havrankova *et al.* (1978) and Eng & Yalow (1981) and to characterize any insulin-like material by h.p.l.c. in combination with radioimmunochemical methods. These studies were carried out along with experiments to demonstrate the production of insulin-like material by mouse foetal brain cells in culture. The presence of somatostatin-like material by these cultures was also investigated, in view of an earlier report by Delfs *et al.* (1980) and

Abbreviations used: h.p.l.c., high performance liquid chromatography; TFA, trifluoroacetic acid; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulphonyl fluoride; PBS (phosphate-buffered saline) consisted of 10mM-sodium phosphate buffer, pH 7.5, containing 0.15M-NaCl.

as a means of further characterizing the cell culture preparation.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM, cat. no. 430-2100), foetal bovine serum, horse serum and sodium bicarbonate were all obtained from Gibco (N.Z.), Auckland, New Zealand. Benzylpenicillin sodium B.P. and streptomycin sulphate B.P. were supplied by Glaxo, Greenford, Middx., U.K.

Monocomponent pig insulin crystals [lot no. 5831160; biological potency 26.8 i.u./mg (50 mg)] and freeze-dried rat insulin [lot no. R810611; biological potency 24.5 i.u./mg (100 µg in 1 mg of human albumin)] were purchased from the Novo Research Institute, Bagsvaerd, Denmark and supplied by Pharmaco N.Z., Auckland, New Zealand. An additional sample of authentic rat insulin was given by Dr. R. Chance of Eli Lilly, Indianapolis, IN, U.S.A. Antiserum GP11 was kindly donated by the Department of Endocrinology, Auckland Hospital. This was raised in a guinea pig to Novo 'Actrapid' insulin (pig) emulsified in Freund's complete adjuvant.

Somatostatin antiserum (SRIF 693) was kindly donated by Dr. S. Reichlin, New England Medical Center Hospital, Boston, MA, U.S.A., and Dr. Jean Rivier of the Salk Institute, La Jolla, CA, U.S.A. generously provided initial samples of somatostatin.

Tetanus toxin and rabbit tetanus antitoxin (100 antitetanus toxin units/ml) were gifts from Dr. I. Batta and Dr. R. O. Thompson, of the Wellcome Research Laboratories, Beckenham, Kent, U.K.

Fluorescein-labelled goat anti-(rabbit γ -globulin) was purchased from Wellcome Reagents, Beckenham, Kent, U.K.

Cytosine-1- β -arabinofuranoside (hydrochloride), poly(D-lysine) hydrobromide, type 1-B, trifluoroacetic acid, no. T-6508, bovine serum albumin (fraction V and radioimmunoassay grade), Norit-A activated charcoal, somatostatin-(1-14) and [Tyr¹]somatostatin were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. A 20% (w/v) solution of bovine serum albumin was supplied by Armour Pharmaceutical Co. Sephadex G-50 was obtained from Pharmacia, Uppsala, Sweden. Protargol (1%; silver-protein, Bodian-Färbung Chroma Gesellschaft) was supplied by Schmid & Co., Stuttgart, Germany.

Na ¹²⁵I (13-17 mCi/µg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Acetonitrile (h.p.l.c. grade) and Sep-Pak C₁₈-silica cartridges were from Waters Associates, Amherst, MA, U.S.A.

Insulin radioimmunoassay

Insulin was iodinated by the method of Greenwood *et al.* (1963) and purified using a cartridge of C₁₈-silica. This was wetted with TFA/water (1:99, v/v) (10 ml), primed with 100 mg of bovine serum albumin, and washed with 10 ml of acetonitrile/TFA/water (50:1:49, by vol.) followed by 10 ml of TFA/water (1:99, v/v). The iodination reaction mixture was applied and unreacted iodide was washed through with 2 × 4 ml of TFA/water (1:99, v/v). 'Damaged' peptide was eluted with 2 ml of acetonitrile/TFA/water (25:1:74, by vol.), and ¹²⁵I-labelled insulin was eluted with 4 × 0.5 ml of acetonitrile/TFA/water (50:1:49, by vol.). Samples containing 2 × 10⁶ c.p.m. (660 kBq) were diluted to 1 ml in assay buffer [50 mM-sodium phosphate (pH 7.5)/5 mM-EDTA/0.02% NaN₃, containing 0.3% bovine serum albumin (fraction V)] and stored at -20°C. Prior to use, monomeric insulin was isolated by gel-filtration on a column (0.9 cm × 50 cm) of Sephadex G-50 equilibrated and eluted in assay buffer without Phenol Red. Precise quantification of pig insulin was carried out by amino acid analysis. Concentrations of insulin in experimental samples are stated in terms of rat or pig insulin as indicated in the text.

Insulin determinations were carried out in triplicate. After a 16-20 h preincubation of sample and antiserum the assay was incubated for a further 3 days with ¹²⁵I-labelled insulin. Antibody-bound ¹²⁵I-labelled insulin was precipitated with 18% poly(ethylene glycol) in PBS.

The composition of the radioimmunoassay mixture was 100 µl of assay buffer, 100 µl of extract or insulin standard, 100 µl of antiserum diluted in assay buffer (final dilution 1:175 000) and ¹²⁵I-labelled insulin (5000 c.p.m., 167 Bq). The assay had a detection limit of 5 pg of insulin.

Somatostatin radioimmunoassay

Concentrations of somatostatin in cultured brain cells and column fractions were determined by radioimmunoassay based on the method used by Patel & Reichlin (1979).

Radioactivity was measured in a Packard Auto-gamma scintillation spectrometer (model 5260) or a Searle automatic gamma counter (model 1195) at efficiencies of 51% and 49%, respectively. Results were calculated using a computer program developed by Dr. G. E. Chapman which fitted a standard curve of percentage bound against concentration of displacing hormone using a four-parameter logistic model.

High performance liquid chromatography

Insulin-like material extracted from rat brain and insulin- and somatostatin-like immunoreactivity produced by brain cell cultures were further

characterized by h.p.l.c. The system (Waters Associates) comprised two solvent delivery pumps (models 6000A and M-45), a sample injector valve, U6K fitted with a 2ml loading loop, a system controller (model 720) and an absorbance detector (model 441). Peptides were detected by their absorbance at 214nm. All separations were carried out at room temperature using a Radial-Pak μ Bondpak C₁₈ cartridge (10 μ m, 8mm $\times$ 100mm) radially compressed in a Z-module. One solvent system was used throughout; solvent A consisted of TFA/water (1:999, v/v) and solvent B consisted of acetonitrile/TFA/water (800:1:199, by vol.). Water was purified by using a Milli-Q system (Millipore) and all solvents were filtered through a 0.22 μ m Durapore filter (Millipore). Solvents were degassed under reduced pressure for 20min before use.

Extracts were pumped onto the column previously equilibrated with solvent A/solvent B (9:1, v/v) at 1.5ml/min using a spare solvent port on the M6000 pump. After re-equilibration (as judged by the absorbance trace at 214nm) for 10min, a 1h linear gradient was pumped onto the column at 1.5ml/min to give a final solvent composition of solvent A/solvent B (3:7, v/v). Fractions (1ml) were collected into 200 μ g of bovine serum albumin (radioimmunoassay grade) from the start of the gradient. The h.p.l.c. column was calibrated with authentic rat and pig insulin.

Extraction and characterization of immunoreactive insulin from rat brain

Extractions of rat brains were carried out essentially as described by Havrankova *et al.* (1978) and Eng & Yalow (1981).

Recoveries of insulin during the procedure were monitored by the addition of ¹²⁵I-labelled insulin (2000–3000c.p.m., 66–100Bq) after homogenization of the brain tissue. Radioactivity which remained at the end of the extraction procedure was used as a measure of recovery. Our recoveries were lower than those reported using the Eng & Yalow (1981) procedure as the Sep-Pak eluates had to be freeze-dried prior to radioimmunoassay.

For subsequent analysis by h.p.l.c., three brains were extracted by either procedure. The extraction method of Havrankova *et al.* (1978) was modified by the removal of bovine serum albumin from the (NH₄)₂CO₃. Extracts from both procedures were diluted with solvent A/solvent B (9:1, v/v) and pumped directly onto the column. Conditions for h.p.l.c. were as described above.

Culture of mouse foetal brain cells

Mouse foetal brain cells were cultured by using the procedure of Sotelo *et al.* (1980) modified as follows.

Culture media no. 1 and no. 2 did not contain crystalline insulin. Medium no. 3 consisted of medium no. 1 without serum components.

The cell suspension was prepared as described by Sotelo *et al.* (1980) using CD-1 mouse embryos at day 12 of gestation. Cells were suspended in 1ml of medium per foetal brain and 0.5ml aliquots were added to 58mm $\times$ 15mm (Nunc) sterile petri dishes which contained 6ml of medium. Cells required for microscopic analysis were grown in dishes containing sterile glass coverslips. Prior to use these dishes were coated by the addition of 0.5ml of a sterile solution (0.1mg/ml) of poly(D-lysine). Cultures required for biochemical analysis were grown directly on plastic dishes.

The cells were incubated (National gas exchange incubator) in a humidified atmosphere of CO₂/air (1:9) at 37°C for 6 days with no medium changes and without disturbing the cells. On day 7 the medium was removed and replaced by medium no. 2. This medium remained on the cells for a further 6 days. On day 13 medium no. 2 was replaced by medium no. 3. Depending on the particular experiments being undertaken, medium no. 3 was replaced at regular intervals or left on the cells for long periods of time.

Phase contrast microscopy

The cultures were examined at different stages of growth using an Olympus CK inverted microscope with phase contrast attachment (Olympus CK-PC). Photographs were taken with a Zeiss C35 camera using Ilford Pan 5 fine-grain black and white film (ASA 50, DIN 18).

Silver staining of neuronal cells

Cells grown on coverslips were stained using Bodian's silver stain (Bodian, 1936) as modified by Gambetti *et al.* (1981). Non silver-stained cells were counter-stained by dipping the slide several times in Aniline Blue.

Cells were viewed under a Zeiss stereo microscope and photographs were taken with a Zeiss MC 63 photomicrographic camera using Agfa-chrome Professional 50S colour film (18 DIN/50 ASA).

Determination of the binding of tetanus toxin to neuronal cells by indirect immunofluorescence

Indirect immunofluorescent identification of neurones using tetanus toxin was carried out by using the method of Mirsky *et al.* (1978).

Extraction and concentration of cultured brain cells and media prior to analysis by radioimmunoassay and h.p.l.c.

Extraction of cells. For each determination, cells were removed from five culture dishes (originally

derived from 2.5 foetal brains) using ice-cold PBS. The cells were centrifuged (bench centrifuge; 1100g, 1 min) resuspended in ice-cold PBS, and re-centrifuged (Beckman Microfuge, 30s). The pellet was homogenized in 1 ml of ice-cold 5M-acetic acid/0.25% bovine serum albumin (radioimmunoassay grade)/1 mM-PMSF using a hand-held glass homogenizer. The homogenate was centrifuged at 10000g for 30 min at 4°C, the supernatant was removed, diluted 3-fold in distilled water, and freeze-dried. Prior to radioimmunoassay, the freeze-dried cell extracts were resuspended to 0.5–1.0 ml of assay buffer.

Extraction of culture medium. Culture medium from brain cell cultures was concentrated using C₁₈-silica (Sep-Paks). The C₁₈ Sep-Pak was wetted with 10 ml of solvent B and excess solvent was removed with 10 ml of solvent A. Culture medium from five dishes of cells was extracted with 6 ml of 5M-acetic acid containing 1 mM-PMSF and applied to the C₁₈ Sep-Pak. This was washed with 10 ml of solvent A followed by 4 ml of acetonitrile/TFA/water (250:1:749, by vol.) and eluted with 4 ml of acetonitrile/TFA/water (500:1:499, by vol.). Fractions were lyophilized in the presence of 200 µg of bovine serum albumin (radioimmunoassay grade) and resuspended in 0.5 ml of assay buffer for radioimmunoassay. When medium from 10 dishes of cells was concentrated, the method was scaled up accordingly and two Sep-Paks were connected in series.

For analysis of insulin (and somatostatin) immunoreactivity by h.p.l.c., culture medium from 10 dishes of cells (60 ml) was applied to two Sep-Paks connected in series. The acetonitrile/TFA/water (500:1:499, by vol.) eluate was diluted 5-fold with solvent A and pumped onto the h.p.l.c. column. Conditions for h.p.l.c. were as described above.

When concentrated culture medium was used, it was necessary to dilute samples before determination of somatostatin. Samples of unconcentrated culture media were acidified to pH 2.0 with solvent A and applied to the column. Authentic somatostatin-(1–14) (5 ng, 100 µl) was also chromatographed and its retention time was calculated after location by radioimmunoassay.

Results

Dissociated brain cells from 12-day-old mouse foetal brains were cultured by the method of Sotelo

et al. (1980) with two modifications: insulin was omitted from the culture medium and after 12 days in culture cells were grown in DMEM without serum components. These modifications did not appear to affect the growth and development of the cells on microscopic examination before and after staining. Following 12 days in culture, medium containing arabinosylcytosine, an inhibitor of DNA synthesis, was replaced with serum-free medium without inhibitor. At this stage, process-bearing cells were well differentiated with large phase-bright cell bodies and extensive networks of processes (Fig. 1a). When the cells were stained with Bodian's silver stain after 15 days in culture, 'neurones' were grouped mainly in clusters interconnected by long straight processes (Fig. 1b). Neurones were also identified in 19-day cultures by indirect immunofluorescence after reaction with tetanus toxin (Fig. 1c). These results were similar to those obtained by phase-contrast and silver impregnation, enabling identification of bipolar rounded neurones and multipolar pyramidal shaped neurones interconnected by long straight processes. Fine networks of neurites, some not visible by phase-contrast microscopy, were also seen. The culture preparation maintained a similar appearance for relatively long periods (20–30 days) in serum-free media (Fig. 1d).

Use of serum-free media without added insulin enabled investigation of the possible production of insulin-like material by brain cell cultures. In initial work, immunoreactive insulin could not be detected by radioimmunoassay in unconcentrated culture media. However, when media from five dishes of cells (30 ml) was concentrated 60-fold by using C₁₈-silica, significant amounts of insulin could be detected. Peak production of immunoreactive insulin (610 pg/30 ml) occurred 14–18 days after changing from serum-free media (Fig. 2). No significant amounts of insulin-like material could be detected after acid extraction of cells pooled from five culture dishes.

The concentration of somatostatin was also determined in the cell culture medium used in the experiment illustrated in Fig. 2. This was carried out partly as a control, because somatostatin is a well-characterized neuropeptide that was shown to be produced by rat cortical neurones in culture (Delfs *et al.*, 1980). The amount of somatostatin was sufficiently high to allow detection without concentration and the amounts in the medium (1.5 ng/6 ml per dish) remained relatively constant throughout

Fig. 1. Characterization of mouse foetal brain cell cultures

(a) Cells after 12 days in culture viewed by phase contrast microscopy (37×). (b) Neurones impregnated with Bodian's silver stain after 15 days in culture (155×). (c) Neurones identified by indirect immunofluorescence following reaction with tetanus toxin (110×). (d) Cells after 30 days of culture viewed by phase contrast microscopy (37×). All procedures were carried out as described in the text.

Fig. 2. Production of insulin-like (○) and somatostatin-like (●) material by cultured mouse foetal brain cells. For insulin determinations culture media from five dishes of cells (maintained on medium no. 3) were pooled, mixed with 6 ml of ice-cold 5 M-acetic acid/1 mM-PMSF and frozen at -80°C until concentration. The acetonitrile/TFA/water (500:1:499, by vol.) eluate from the C_{18} Sep-Pak was freeze-dried in the presence of 200 μg of bovine serum albumin (radioimmunoassay grade) and resuspended in 0.5 ml of assay buffer for radioimmunoassay. Values are not corrected for the 'apparent' insulin-like material in concentrated medium no. 3 which did not contain cells (0.06 ng/30 ml of culture medium). Samples of unconcentrated media from the same preparation of cells were taken and stored at -80°C prior to somatostatin determinations.

the time-course of the experiment. No detectable somatostatin-like material could be extracted from cells maintained in serum-free media for less than 12 days. Even at this time, amounts extracted from the cells were approx. 50-fold lower than the concentration present in the medium (results not shown).

Insulin- and somatostatin-like material produced by cultured brain cells was further characterized by h.p.l.c. Fig. 3 shows the elution profile of insulin-like material produced by brain cells cultured in serum-free media for '6' (5 and 7 day pooled samples) and 27 days, respectively. In both cases two main peaks of immunoreactive insulin were identified. The earlier peak had a slightly later retention time, whereas the major peak of immunoreactive material was eluted considerably later than authentic rat insulin. Fig. 4 shows the results of h.p.l.c. analyses of immunoreactive somatostatin present in the culture medium after '6', 27 and 40 days in culture. In all cases, multiple peaks of somatostatin immunoreactivity were resolved by h.p.l.c.

The concentration of insulin in brain is a matter of debate and for this reason, attempts were made

Fig. 3. Comparison by h.p.l.c. of insulin-like material released into the medium by cultured mouse neuronal cells with that extracted from whole rat brain.

Two 30 ml pooled samples of media from cells maintained for 5 and 7 days without serum and 60 ml of media from cells maintained for 27 days without serum were concentrated with two C_{18} Sep-Paks connected in series. The acetonitrile/TFA/water (500:1:499) eluate from the C_{18} Sep-Paks was diluted 5-fold with solvent A, and subjected to h.p.l.c. Fractions (1.05 ml) were collected into 200 μg of bovine serum albumin (radioimmunoassay grade), freeze-dried and resuspended in 0.5 ml of assay buffer for radioimmunoassay. The Figure shows the distribution of insulin-like immunoreactivity (a) '6' days and (b) 27 days after removal of serum components. The arrow (↓) indicates the elution position of 5 ng of rat pancreatic insulin (retention time 39.0 ± 0.7 min). (c) Shows a representative elution profile of immunoreactive insulin extracted from three adult rat brains by the procedure of Havrankova *et al.* (1978).

Fig. 4. Characterization by h.p.l.c. of somatostatin-like material released into the medium by cultured brain cells

Samples of cell culture media were taken at three different time intervals for h.p.l.c. Unconcentrated culture medium (40 days) was assayed without dilution. The Figure shows the distribution of somatostatin-like immunoactivity at (a) '6' days, (b) 27 days, and (c) 40 days after removal of serum components. The elution profile of somatostatin-(1-14) (5ng) chromatographed for reference is shown in the upper portion of (a).

tracted by two different procedures [Havrankova *et al.* (1978); Eng & Yalow (1981)]. In terms of rat insulin standard, approx. 2.5 times (range 1.5–4.94) more insulin was extracted by the procedure of Havrankova *et al.* (1978) than that of Eng & Yalow (1981) after correction for the recovery of ^{125}I -labelled insulin (Table 1). Higher concentrations of insulin-like material in Havrankova *et al.* (1978) extracts were also obtained by using pig insulin standard (3.61-fold). Mean plasma insulin concentrations were very similar for rats used in both the Havrankova *et al.* (1978) and Eng & Yalow (1981) procedures, being 0.41 and 0.45 ng of pig insulin/ml, respectively.

Insulin-like material extracted from rat brain by both methods was further characterized by h.p.l.c. Initially, multiple peaks of immunoreactive insulin were observed after h.p.l.c. analysis of the Havrankova *et al.* (1978) extract. In subsequent extractions, and in both extracts using the Eng & Yalow (1981) method, only a single peak of immunoreactivity was observed which had a very similar elution position to that of authentic rat insulin. For comparison with insulin-like material produced by brain cell cultures, a representative elution profile of insulin extracted from whole adult rat brain by the method of Havrankova *et al.* (1978) is shown in Fig. 3.

Discussion

Dissociated brain cells were found to grow and differentiate in culture in the absence of added pancreatic insulin; examination by phase-contrast microscopy, silver impregnation and immunofluorescence following reaction with tetanus toxin revealed that a high proportion resembled neurones. The results were similar to those reported by Sotelo *et al.* (1980) for brain cell cultures with insulin supplements. In addition, the appearance of our cells was not markedly changed by longer-term culture in serum-free media.

Concentrations of peptide hormones measured in cells or media are reported in terms of number of culture dishes or volume of media. Quantification based on cell numbers was not possible because of the conditions of culture. Determinations of protein concentration were also unsatisfactory because available methods were too insensitive.

When medium from cultured mouse foetal brain cells was concentrated on C_{18} -silica and examined by h.p.l.c. in conjunction with radioimmunoassay, two main forms of insulin-like material were apparent (Fig. 3). The major peak of insulin-like material differed markedly from authentic rat insulin. By comparison, the retention time of the first peak was only slightly longer than that of the standard. Although authentic mouse pancreatic

to extract immunoreactive insulin from rat brain and compare it with insulin-like material produced by brain cells in culture. Whole rat brain was ex-

Table 1. *Extraction of insulin-like material from rat brain using the procedures of Havrankova et al. (1978) and Eng & Yalow (1981)*

Nine separate brain extractions were carried out by the method of Havrankova *et al.* (1978), and five extractions by the procedure of Eng & Yalow (1981). Freeze-dried extracts from both procedures were resuspended in 2 ml of 50 mM-sodium phosphate buffer, pH 7.5. Plasma and brain concentrations, given in terms of pig and rat insulin, were quantified by radioimmunoassay. The average rat brain weight was 2.05 ± 0.019 g ($n = 14$, mean $\pm$ S.E.M.). Brain insulin concentrations were corrected for recovery of ^{125} I-labelled insulin at the end of the extraction procedure. All values are means $\pm$ S.E.M. for the number of experiments shown in parentheses.

Extraction method	Average recovery of ^{125} I-labelled insulin (%)	Rat brain insulin concentrations (ng of insulin/g wet wt.)		Plasma insulin (ng of pig insulin/ml)
		Pig insulin equivalents	Rat insulin equivalents	
Havrankova <i>et al.</i> (1978)	30.0 ± 4.1 (9)	1.48 ± 0.12 (9)	2.33 ± 0.34 (5)	0.41 ± 0.11 (5)
Eng & Yalow (1981)	26.2 ± 0.5 (5)	0.41 ± 0.13 (5)	0.90 ± 0.36 (5)	0.45 ± 0.12 (5)

insulin was not available for comparison it is unlikely that these differences in chromatographic behaviour could be attributed to species variation. Pig and rat insulin were only partially separated by h.p.l.c. (results not shown) and their primary sequences differ much more than those of the rat and mouse hormones (Brown *et al.*, 1955; Bünzli *et al.*, 1972; Blundell & Humbel, 1980).

Further characterization of the two main forms of immunoreactive insulin-like material was hampered by the small amounts present and by the logistical difficulties of large-scale culture of mouse foetal brain cells. Insulin-like growth factors have been demonstrated in cultures of rat brain and anterior pituitary (Binoux *et al.*, 1981) but the lack of cross-reactivity between these factors and antisera raised to pancreatic insulin (Blundell & Humbel, 1980) renders this possibility unlikely. Further studies are clearly needed, including investigation of the interaction between the putative insulin and insulin receptors.

The production of multiple forms of insulin-like material by mouse foetal brain cells in culture is of particular interest in view of the differences in concentrations of insulin in rat brain reported by Havrankova *et al.* (1978, 1979) and Eng & Yalow (1981). Early reports by the former group suggested that insulin was present in rat brain in amounts 25 times greater than that in plasma. This value was subsequently revised to 8 ± 2 ng of pig insulin equivalents/g wet weight (Rosenzweig *et al.*, 1980). In contrast, Eng & Yalow (1980) reported concentrations generally less than that in plasma.

In the present investigations, extracts of whole rat brain were carried out using the experimental protocols of each group. While the absolute concentration of insulin was significantly lower than that reported by Havrankova *et al.* (1978), about 2.5 times more insulin was extracted by this procedure than by that of Eng & Yalow (1981).

Moreover, the insulin concentration present in rat brain, determined after extraction by the Havrankova *et al.* (1978) procedure and after correction for the recovery of ^{125} I-labelled insulin, was approx. 3-fold higher than the plasma concentration (Table 1). Rats used for both extraction procedures had very similar concentrations of plasma insulin. It is difficult to reconcile the differences in yields of insulin extracted by procedures which are in truth very similar. Eng & Yalow (1981) suggested that higher concentrations of insulin may result from contamination. As a precaution we followed their advice and all glassware used for extraction was autoclaved before use. It is possible that lower yields given by the Eng & Yalow (1981) procedure were due to the failure of some material to adsorb to or be eluted from the Sep-Paks.

In an attempt to characterize insulin-like material extracted from rat brain by each procedure, samples were analysed by h.p.l.c.

The similarities in chromatographic behaviour of insulin extracted from rat brain by either procedure and authentic rat pancreatic insulin are consistent with the similar immunological properties reported previously (Havrankova *et al.*, 1978; Eng & Yalow, 1981) and those found in the present study (results not shown).

There are clear differences between insulin-like material extracted from rat brain and that produced by mouse foetal brain cells in culture. The multiple forms of immunoreactive insulin produced by brain cells in cultures may be specific to foetal tissue and the possibility of a role for insulin-like material in brain maturation should not be neglected. Alternatively, other forms of insulin may not readily be extracted from rat brain by the procedures used and it is possible that the multiple forms of insulin-like material produced by the brain cells could result from incomplete processing of a precursor molecule. However no product-pre-

cursor relationships were apparent following analysis of medium taken from cells at different stages of culture. It is also conceivable that this heterogeneity could result from some as yet undefined chemical modification.

It is well established that added pancreatic insulin has a wide range of metabolic effects in brain tissue. Immunoreactive insulin has been located in nerves to skeletal muscles of the cat and is released upon excitation of nerves (Uvnäs & Uvnäs-Wallenstein, 1978). Insulin receptors have also been located on synaptosomes (Pacold *et al.*, 1978). The possibility must also be entertained that insulin synthesized within brain cells may exert effects on general metabolism within the central nervous system.

Somatostatin is a well characterized neuropeptide and its production by cultured mouse foetal brain cells was studied partly as a control to enable further characterization of the preparation. In contrast to the production of insulin-like material the concentration of somatostatin in the medium remained relatively constant (1.5 ng/6 ml per dish) over the time period studied (Fig. 2).

Our experiments confirmed an earlier report by Delfs *et al.* (1980) who described the production of somatostatin by rat cortical neurones in culture with a 6-fold higher concentration of somatostatin in the cells than in the medium. However we could not detect somatostatin in the cells until after 12 days in culture in serum-free media. Even at this time the concentration in the cells was much less than that in the medium. When serum-free medium was used for the culture of chick embryo cerebral hemispheres, somatostatin was found in higher concentrations in the medium than in the cells (Dray *et al.*, 1982). The lower figures for somatostatin in medium reported by Delfs *et al.* (1980) may be due to the different species employed or the presence of proteinases in serum added to their culture system.

Multiple forms of immunoreactive somatostatin were found in the medium at all time periods studied. There was a trend for the late-eluting material present in the '6' day sample (Fig. 4) to diminish with increasing periods of culture in serum-free media. In other respects there were no obvious product-precursor relationships. Some of the immunoreactive material was eluted at the same position as synthetic somatostatin-(1-14) used as a reference. Three different molecular weight forms of somatostatin have been shown to be present in rat brain (Patel *et al.*, 1981). These correspond to somatostatin-14, -28 and 14K. Immunoreactive somatostatin, similar to synthetic somatostatin-(1-14) and somatostatin-28 has been found both in foetal rat brain cells (Peterfreund & Vale, 1982) and in chick embryo cerebral hemi-

spheres (Dray *et al.*, 1982). It is likely that these forms of somatostatin are also produced by mouse foetal brain cells in culture.

The production of insulin- and somatostatin-like immunoreactive material by mouse foetal brain cells in culture suggests that the system may be of value for the study of neuropeptide biosynthesis.

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References

- Binoux, M., Hossenlopp, P., Lassarre, C. & Hardouin, N. (1981) *FEBS Lett.* **124**, 178-184
- Blundell, T. L. & Humbel, R. E. (1980) *Nature (London)* **287**, 781-787
- Bodian, D. (1936) *Anat. Rec.* **65**, 89-97
- Brown, H., Sanger, F. & Kitai, R. (1955) *Biochem. J.* **60**, 556-565
- Bünzli, H. F., Glatthaai, B., Kunz, P., Mulhaupt, E. & Humbel, R. E. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 451-458
- Delfs, J., Robbins, R., Connolly, J. L., Dichter, M. & Reichlin, S. (1980) *Nature (London)* **283**, 676-677
- Dray, F., Louis, J. C., Rougeut, C., Mandel, P. & Bennett, C. (1982) *FEBS Lett.* **145**, 123-127
- Eng, J. & Yalow, R. S. (1980) *Diabetes* **29**, 105-109
- Eng, J. & Yalow, R. S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4576-4578
- Eng, J. & Yalow, R. S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2683-2685
- Gambetti, P., Autilio-Gambetti, L. & Papasozomenos, S. Ch. (1981) *Science* **213**, 1521-1522
- Greenwood, F. C., Hunter, W. H. & Glover, J. S. (1963) *Biochem. J.* **89**, 114-123
- Havrankova, J., Schmechel, D., Roth, J. & Brownstein, M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5737-5741
- Havrankova, J., Roth, J. & Brownstein, M. J. (1979) *J. Clin. Invest.* **64**, 636-642
- Mirsky, R., Wendon, K. M. B., Black, P., Stolkin, C. & Bray, D. (1978) *Brain Res.* **148**, 251-259
- Pacold, S. T., Small, E. & Blackard, W. G. (1978) *Clin. Res.* **26**, 34a (abstract)
- Patel, Y. C. & Reichlin, S. (1979) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M. & Behrman, H. R., eds.), pp. 77-99, Academic Press, New York, San Francisco, London
- Patel, Y. C., Wheatley, T. & Ning, C. (1981) *Endocrinology* **109**, 1943-1949
- Peterfreund, R. A. & Vale, W. (1982) *Brain Res.* **239**, 463-477
- Rosenzweig, J. L., Havrankova, J., Lesniak, M. A., Brownstein, M. & Roth, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 572-576
- Sotelo, J., Gibbs, C. J., Jr., Gajdusek, D. C., Toh, B. H. & Wurth, M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 653-657
- Uvnäs, B. & Uvnäs-Wallenstein, K. (1978) *Acta Physiol. Scand.* **103**, 346-348