Journal of Chromatography, 357 (1986) 139–146
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 414

CHROMATOGRAPHIC CHARACTERIZATION OF ADULT AND FOETAL RAT INSULIN

M. DE GASPARO and M. FAUPEL*
Biological and Chemical Research Laboratories, Pharmaceuticals Division, Ciba-Geigy Limited, CH-4002 Basle (Switzerland)
(Received December 12th, 1985)

SUMMARY

Foetal rat pancreatic rudiments explanted on day 14 of gestation were grown in organ culture in medium enriched with amino acids. The size of the insulin granules was increased, resulting in an insulin granule volume fraction greater than the volume fraction measured in pancreas grown *in vivo*. The pancreas was extracted and the insulin compared. Serial dilution curves of extracts of adult pancreas and pancreas grown *in vitro* are parallel in the insulin radioimmunoassay, whereas extracts of pancreas of foetus developing *in utero* appear immunologically different. Adult and foetal rat insulin (*in utero*) were purified using chromatography on OPTI UP C12, cellulose thin-layer chromatography plates, cellulose acetate foil electrophoresis and finally high-performance liquid chromatography. The ratio of insulin I to insulin II was found to be 1.5 for the adult and 2.7 for the foetus. These results show that there is an unequal expression of the two non-allelic genes controlling insulin biosynthesis in foetal and adult rat pancreas.

INTRODUCTION

Foetal rat pancreases dissected at 14 days and grown *in vitro* in a medium enriched with amino acids contain B granules of greater size than pancreases of the same age grown *in utero*[1]. The insulin granule volume represents 17.6% of the total volume of the B cell, whereas it is only 10.8% *in vivo*. This observation is characteristic of the insulin-producing cells, as the glucagon and the zymogen granules are of comparable size *in vitro* and *in vivo*. However, the mean insulin content of the B cell surprisingly is six times lower in culture than *in vivo*, when measured with a standard insulin radioimmunoassay.

Several hypotheses have been advanced to explain this observation[1]. There could be a marked difference in the granular concentration of insulin between granules qualitatively similar in morphology, or there could be more extragranular insulin *in vivo*. The insulin to proinsulin ratio may also differ, leading to underestimation of the insulin concentration when an antibody reacting preferentially with insulin is used. Finally, what is measured in the foetal pancreas grown *in vitro* may be fun-

0021-9673/86/$03.50

damentally different from what is detected in the pancreas of the foetus growing *in utero*.

Therefore, foetal pancreases developing *in vitro* and *in vivo* were extracted, and dilutions of the extracts were compared in the radioimmunoassay to insulin from adult rat pancreas and crystalline rat insulin. Similar extracts were also purified and submitted to electrophoresis for comparison. The results show that there is an unequal expression of the two non-allelic genes controlling insulin biosynthesis in foetal and adult rat pancreas and that insulin from the foetal pancreas, which has differentiated *in vitro*, is radioimmunologically similar to adult insulin.

EXPERIMENTAL

Male and female rats [Tif: RAI f(SPF)], fed *ad libitum*, were mated. For the determination of gestational age, fertilization (day 0 of embryonic life) was assumed to have occurred at noon on the day where the vaginal plug was discovered. Foetal rat pancreases were removed on day 14 of gestation and cultured for 6 days on Millipore filter platforms over polystyrene culture wells, as described previously[2]. The culture medium was based on Basal Eagle's Medium supplemented with "physiological" amino acids to a final concentration seven times greater. After 6 days of culture, with a daily change of medium, the pancreatic rudiments and pancreases from foetuses killed on day 20 of gestation were collected, frozen on dry-ice and stored at −80°C until assayed. The pancreas of the mother was also dissected and processed similarly.

Serial dilution

Foetal pancreases were sonicated in acid–ethanol and serially diluted in the range of 0.6–6 ng/ml with 0.2 *M* glycine buffer (pH 8.8). Adult pancreases from pregnant rats were extracted with acid–ethanol according to Davoren[3] and diluted in the same way. The dilutions were assayed using the insulin radioimmunoassay of Herbert *et al.*[4].

The calibration graph for crystalline rat insulin (Novo RC 791009) and antibodies insulin (Pentex Batch 2) was calculated with a Hewlett-Packard 9815 desk calculator after logit (B/B_0)/log transformation. The insulin contents of the various dilutions of the extracts were measured on this calibration graph after similar mathematical treatment. The regression line between log (dilution) and log (insulin content) was calculated. The slope of the regression line after this transformation was independent of the concentration of the starting material and a perfect correlation between the dilution and the content measured has an angular coefficient of 1.

Foetal tissue grown *in utero* and *in vitro* contained a mean of 466 and 211 ng of insulin, respectively, per pancreas whereas pancreas of pregnant rats contained roughly 145 μg of insulin.

Extraction and purification of insulin (Fig. 1)

Pancreatic tissues was homogenized in ice-cooled 0.1 *N* hydrochloric acid–1% sodium chloride with a Polytron homogenizer at setting 7 for three times 7 s, extracted in hydrochloric acid–sodium chloride at 4°C for 4 h with continuous agitation and centrifuged at 2000 *g* for 30 min. The supernatant was decanted and the tissue

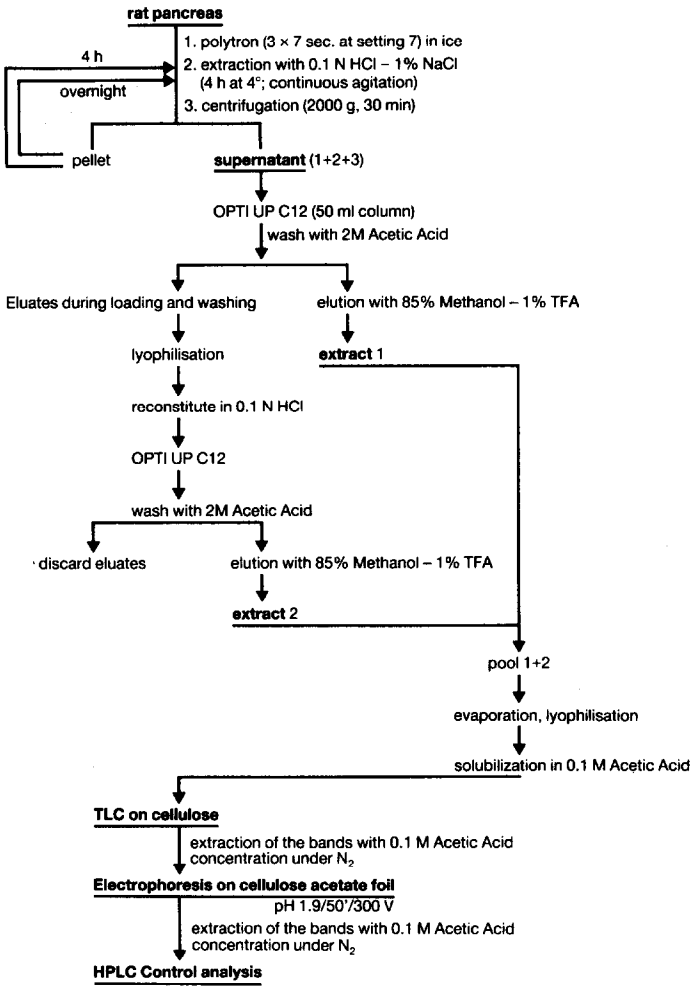


Fig. 1. Extraction and purification procedure.

was re-extracted twice, for 4 h and overnight, respectively. The three extracts were combined and further purified on a 50-ml OPTI UP C12 column (Antecgel dodecyltrichlorosilane, 200–500 μm, 90–125 A; Antec, Bennwil, Switzerland).

The column was washed with 2 *M* acetic acid until the sodium chloride of the extracting fluid was entirely removed (test with silver nitrate) and then eluted with 85% methanol–1% trifluoroacetic acid. The eluate during loading and washing procedures of the column was lyophilized, reconstituted in 0.1 *N* hydrochloric acid–1% sodium chloride, reloaded on the column and washed and eluted as previously. The two eluates were pooled, evaporated, reconstituted with 2 *M* acetic acid and lyophilized. A 3.65-g amount of foetal pancreas and 7.47 g of maternal pancreas gave 6 mg and 47 mg of lyophilizate, respectively.

To purify the extracts further, the lyophilizate was reconstituted in 0.1 *N* acetic acid (2.5 ml for the adult and 0.5 ml for the foetal extract). An aliquot of 40 μl of

each extract was used for preliminary chromatographic studies. Aliquots of 250 μl were linearly applied on a thin-layer chromatographic (TLC) plate (Merck 5718) as a band 14 cm long, using the Linomat III apparatus from Camag (Muttenz, Switzerland). A 5-μl volume of a NOVO rat insulin solution (100 μg in 100 μl of 0.1 *N* acetic acid) was also applied in a 1-cm band on each side of the 14-cm band as a reference standard.

The TLC run was performed in a saturated chamber with pyridine–butanol–amyl alcohol–methyl ethyl ketone–formic acid–acitic acid–water (25:20:15:10:3:3:25). The Novo reference bands were developed with Acid Violet 6B and scanned at 530 nm (Scanner II, Camag). The bands of interest were extracted with 0.1 *N* acetic acid, filtered, centrifuged and concentrated under nitrogen.

The second purification step consisted in electrophoresis on cellulose acetate foils (Innovativ-Labor, Adliswil, Switzerland) at pH 1.9 (93.5 g of formic acid and 26.5 g of acetic acid in 100 ml of water) for 50 min at 300 V. The Novo reference bands were developed with Barton's reagent[5] and scanned at 590 nm. The bands of interest were extracted as before and prepared for high-performance liquid chromatographic (HPLC) analysis, TLC and electrophoresis controls. The last procedure was performed immediately and repeated after 1 month at -10°C.

HPLC was performed using a Spectra-Physics SP 8700 solvent delivery system, a WISP 710 A automatic sample injector (Waters), a Kratos 773 variable-wavelength UV detector set at 220 nm, an HP 3390 A integrator (Hewlett-Packard) and a stainless-steel column (250 × 46 mm I.D.) packed with 10 μm Nucleosil octadecylsilica (Macherey, Nagel & Co.). The solvent for the HPLC included 0.1 *M* sodium dihydrogen phosphate adjusted to pH 2.1 with phosphoric acid (solvent A) and acetonitrile (solvent B). The initial B:A solvent ratio was 25:75 with a flow-rate of 1 ml/min. Later, the proportion of solvent B was increased to 40% during 10 min, and after 20 min it was reduced to 25% during 2 min.

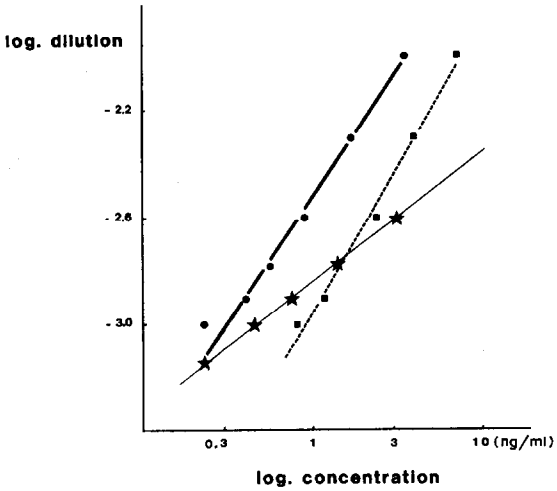


Fig. 2. Insulin radioimmunoassay: serial dilution curve of extracts from adult (●) and foetal pancreas grown *in vitro* (■) or *in utero* (★).

RESULTS

Serial dilution curve

As expected, as rat insulin was used as a standard, extracts from pregnant rat pancreas gave a good correlation between dilution and concentration with a slope close to 1 (1.09 ± 0.03; $n = 20$). The slope of the dilutions of the extract of pancreas grown *in vitro* in enriched amino acids was very similar (0.94 ± 0.01; $n = 30$), whereas there was a very significant change in the angular coefficient with extracts from pancreas developing *in utero* [1.97 ± 0.07; $n = 20$; mean ± standard error of the mean (S.E.M.)] (Fig. 2). Therefore, foetal pancreas grown *in vitro* contains an insulin radioimmunologically similar to the adult insulin, whereas the properties of the insulin synthesized in the pancreas of foetus developing *in utero* appears different.

Insulin extracted from foetal and adult pancreas

The extracts of the pancreas showed two major bands corresponding to the two forms of insulin in the rat. However, the relative proportions of insulin I and II differed in the extracts from the foetal and adult pancreas. On cellulose TLC plates (Fig. 3), the ratio after acid violet treatment was 69:31 (2.2) in the adult, compared with 84:16 (5.25) in the foetus.

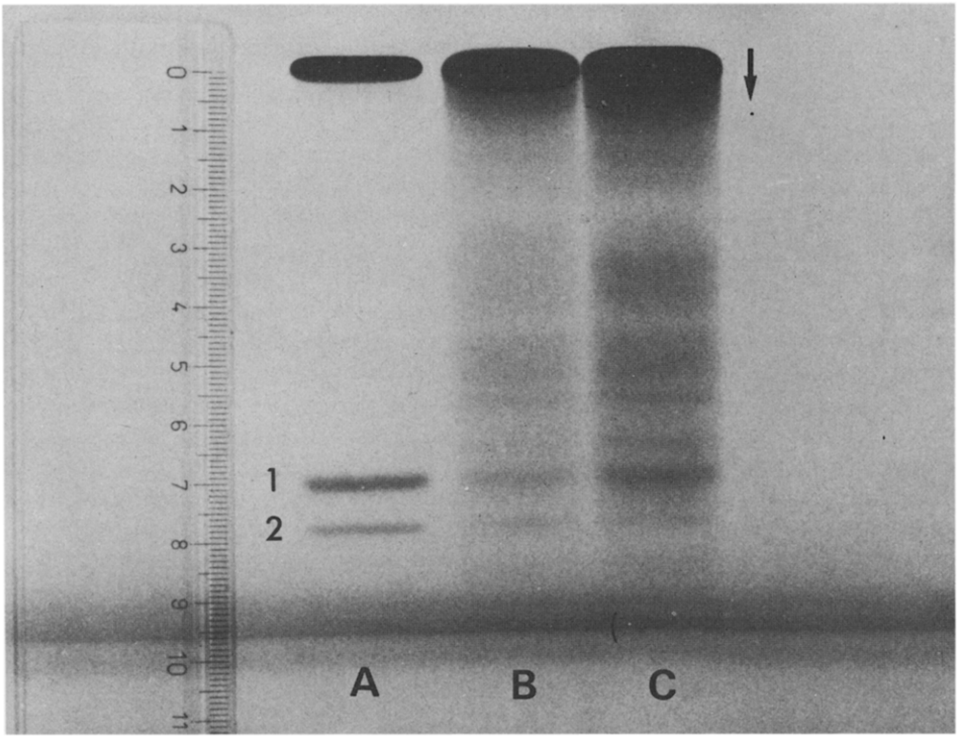
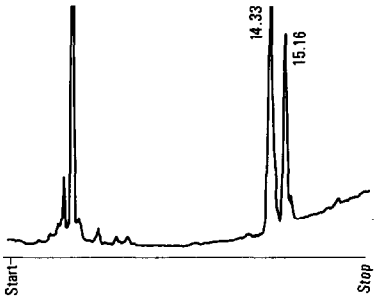


Fig. 3. TLC on a cellulose plate. (A) Novo, rat insulin (1 = insulin I; 2 = insulin II); (B) extracted adult rat insulin; (C) extracted foetal rat insulin after OPTI UP C12 purification. Developing solvent: pyridine–butanol–amyl alcohol–methyl ethyl ketone–formic acid– acetic acid–water (25:20:15:10:3:3:25). Solvent front: 15 cm. Detection: Acid Violet 6B.



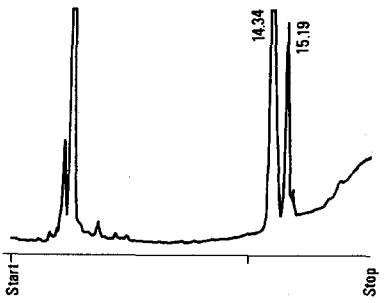


Fig. 4. HPLC of adult rat insulin. Ratio of insulin I to insulin II = 60.74:39.24.

Fig. 5. HPLC of foetal rat insulin. Ratio of insulin I to insulin II = 72.68:27.31.

To eliminate any artefacts of co-chromatography, the measurements were repeated after extraction of the peaks from the cellulose plate and electrophoresis on cellulose acetate foil. Using Barton's reagent, the ratios were 64:36 (1.8) and 75:25 (3.0) for adult and foetal pancreas, respectively. The final HPLC step gave a ratio of pure insulin I to insulin II of approximately 60:40 (1.5) for the adult and 73:27 (2.7) for the foetal pancreatic extract (Figs. 4 and 5).

After 1 month at −10°C, insulin I was still pure and intact, whereas insulin II produced a second peak, probably due to the gradual oxidation of the methionine at position 29 of the insulin B chain. The phenomenon occurred with both adult and foetal insulin II (Figs. 6 and 7).

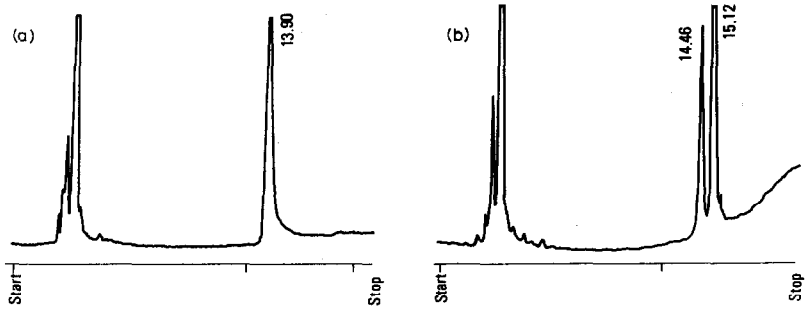


Fig. 6. HPLC of adult rat insulin I (a) and insulin II (b) after 1 month at −10°C.

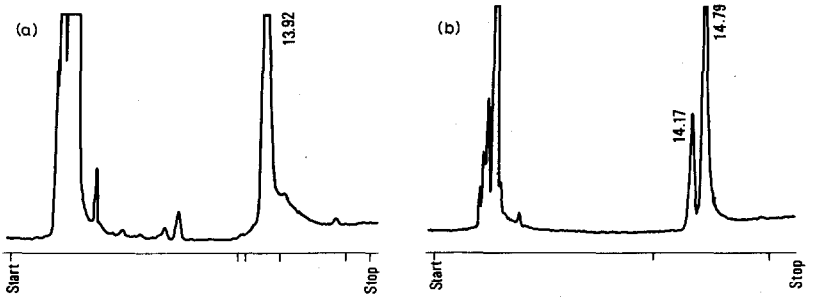


Fig. 7. HPLC of foetal rat insulin I (a) and insulin II (b) after 1 month at −10°C.

DISCUSSION

The quasi-parallelism between the insulin extracted from adult and foetal pancreas differentiating *in vitro* suggests the accumulation of an "adult-like" insulin in the foetus, and a similar insulin to pro-insulin ratio was detected with the antibody used. Therefore, the lower insulin content *in vitro* and the greater insulin granule volume cannot be satisfactorily explained by a difference in the physico-chemical nature of foetal insulin. It is not a fixative artefact, as it is specific for the B granules. Nor can it be a consequence of intragranular zinc depletion[1], as an increased concentration of zinc in the culture medium had no effect on the cellular development of the rudiment, the pancreatic insulin concentration or the granule diameter[1]. It is therefore postulated that the morphological appearance is due to the intragranular accumulation of amino acids in the B cells producing some swelling of the granule, as a consequence of a change in pH and in osmotic pressure.

The absence of parallelism in the radioimmunoassay between adult pancreas and foetal pancreas grown *in utero* could be due to several mechanisms: non-specific immunologically unrelated interfering factors, biologically inactive fragments of insulin, partially denatured insulin or fragments containing the antigenic determinants or other compounds containing regions of homology with the antigenic determinants[6]. Whatever the cause, it is manifested in a different chromatographic ratio between insulin I and II.

It has already been reported that the pancreatic content of insulin I was increased in adult rodents particularly in the pregnant state[7]. This was not confirmed in this study, as the insulin I to insulin II ratio in the extract from pregnant rats was comparable to that found in normal adult rats[7,8] and in the crystalline rat insulin standard. However, the analysis of the extracts described here shows that there was even more insulin I than insulin II in the pancreas of the foetus developing *in utero*, compared with the adult pancreas. The ratio was 2.7 compared with 1.5 after the final purification.

Interestingly, the foetal rat insulin stimulates glucose uptake by the adult diaphragm, whereas adult insulin has no effect on the foetal diaphragm[9]. Moreover, foetal pancreatic insulin is reported to be a monomeric insulin with full biological activity on glucose incorporation into lipid[10]. This may indicate a more important biological role of insulin I in the foetus.

A mechanism by which biosynthesis of insulin I is increased relative to that of insulin II has been suggested to exist both in the adult and in the developing rat pancreas at the level of existing mRNA translation[7]. Our finding in the rat developing *in utero* is analogous to the observation made by Cordell *et al.*[11] in a pancreatic tumour, whereby two-dimensional gel analysis of immunoprecipitated rat I and II preproinsulin synthesized *in vitro* revealed a 10:1 ratio of rat I to rat II proteins. An equivalent amount of both I and II precursor transcript was detected. Therefore, it was postulated that the control consisted in a preferential translation of rat insulin I mRNA with enhanced initiation at the polysomes. A capping defect of rat II mRNA at the 5′ terminus was also discussed[11].

Preferential translation of a gene is not an uncommon observation. In the anglerfish pancreas, the synthesis of one form of pancreatic somatostatin predominates[12]. Moreover, the relative ratio of IGF 2 to IGF 1 is greater in the rat foetus[13].

On the basis of our data on the rat, it seems conceivable that there are two insulins in other species also, one ceasing to be expressed completely at an early stage in foetal life. Rat II insulin gene is of ancestral origin[17] and this form is ontogenically related to mammalian insulin[18]. Our observation in the rat foetus developing *in utero* postulates a set "developmental clock" with a time-switch that decreases insulin I synthesis after birth to basal conditions.

This mechanism does not seem to come into operation when the pancreas differentiates *in vitro*, and the maternal environment therefore appears to be critical for the expression of the genome. The switch appears to be glucose sensitive[14].

In fact glucose is an important modulator of the rate of insulin biosynthesis, through an increase in the initiation of mRNA[15,16]. Therefore, even more insulin I might be produced in the foetus of the diabetic mother. Foetal pancreas and pancreatic tumours[11] show a similarity in that there is an unequal expression of the two non-allelic genes controlling insulin biosynthesis. This tends to confirm that certain forms of cancer are due to some anachronistic autocrine mechanism by which a normally repressed foetal gene is expressed in the adult[19]. It is worth noting that a pancreatic tumour in the adult rat showed the same stage of differentiation as a 19-day-old foetal pancreas[20]. The genetic expression in the tumour is therefore similar to that in the foetus.

ACKNOWLEDGEMENTS

This paper is dedicated to Dr. P. A. Desaulles in appreciation of his continuous scientific interest and support. We also thank Mr. R. Grasser and Mrs. Y. Ledermann for their excellent technical assistance and Mrs. F. Bingert for typing the manuscript.

REFERENCES

1 G. R. Milner, M. de Gasparo, R. Kay and R. D. G. Milner, *J. Endocrinol.*, 82 (1979) 179.
2 M. de Gasparo, G. R. Milner, P. D. Norris and R. D. G. Milner, *J. Endocrinol.*, 77 (1978) 241.
3 P. R. Davoren, *Biochim. Biophys. Acta*, 63 (1962) 150.
4 V. Herbert, K. S. Lau, C. W. Gottlieb and S. J. Bleicher, *J. Clin. Endocrinol.*, 25 (1965) 1375.
5 E. von Arx and M. Faupel, *J. Chromatogr.*, 123 (1976) 439.
6 B. A. L. Hurn and J. Landon, in K. E. Kirkham and W. M. Hunter (Editors), *Radioimmunoassay Methods*, Churchill, London, 1971, p. 121.
7 K. Kakita, K. O'Connell and M. A. Permutt, *Diabetes*, 31 (1982) 841.
8 L. B. Rall, R. L. Pictet and W. J. Rutter, *Endocrinology*, 105 (1979) 835.
9 J. M. Felix, M. T. Sutter, B. Ch. J. Sutter and R. Jacquot, *Horm. Metab. Res.*, 3 (1971) 71.
10 C. Watts and K. R. Gain, *Diabetes*, 33 (1984) 50.
11 B. Cordell, D. Diamond, S. Smith, J. Pünter, H. H. Schöne and H. M. Goodman, *Cell*, 31 (1982) 531.
12 B. D. Noe, *J. Biol. Chem.*, 256 (1981) 9397.
13 P. D. Gluckman and J. H. Butler, *J. Endocrinol.*, 99 (1983) 223.
14 K. Kakita, S. Giddings and M. A. Permutt, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 2803.
15 K. Kikata, S. J. Giddings, P. S. Rotwein and M. A. Permutt, *Diabetes*, 32 (1983) 691.
16 H. Okamoto, *Mol. Cell. Biochem.*, 37 (1981) 43.
17 G. I. Bell, R. L. Pictet, W. J. Rutter, B. Cordell, E. Tischer and H. M. Goodman, *Nature (London)*, 284 (1980) 26.
18 J. L. Clark and D. F. Steiner, *Proc. Natl. Acad. Sci. U.S.A.*, 62 (1969) 278.
19 R. R. Bürk, in G. Fischer and R. J. Wieser (Editors), *Hormonally Defined Media. A Tool in Cell Biology*, Springer, Berlin, 1983, p. 57.
20 V. Iwanij and J. D. Jamieson, *J. Cell Biol.*, 95 (1982) 742.