Establishment of serum based medium essentially free of proteolytic activity for the culture of mouse pancreatic islets[1,2]

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Summary. The present study demonstrates that a) serum based culture medium degrades ^{125}I insulin; b) heat inactivation of serum (1 h, 56 °C) inhibits its proteolytic activity leading to the recovery of more insulin secreted by islets cultured in the presence of high glucose concentration alone or with glucagon; c) aprotinin also favoured the accumulation of secreted insulin by protecting the hormone from a residual degradative capacity of the heat treated serum.

The introduction of Andersson's simple but elegant Petri dish method which involves immersion of isolated mouse islets in culture medium has permitted their long-term culture under controlled conditions[5]. Islets cultured in this system maintain their structural integrity, the ability to synthesize and release insulin[6] as well as to incorporate ^{3}H-thymidine into DNA[7] under glucose stimulation. The presence of calf serum ensures attachment of islets to the bottom of the culture dish, which is essential for their viability. It was found in our laboratory that serum-based culture medium does support cultured islets but has proteolytic activity leading to the destruction of insulin. In pilot studies with untreated serum, it was furthermore found that the insulin degradative capacity of the serum decreased during culture at 37 °C, an observation also made by Andersson et al.[6]. The present paper describes a) the insulin degradative capacity of serum and b) the preparation of a serum based culture medium essentially free of proteolytic activity. This medium maintains islets in good functionnal state assessed by their response to glucose and glucagon.

Material and methods. Isolation of islets of Langerhans and culture technique. Female Swiss mice weighing 25–30 g were starved overnight prior to operative procedures. The pancreata of 3 animals were distended by an injection of Hepes-buffered Hanks solution, pH 7, cut into small pieces and incubated in the presence of 2000 U collagenase (Worthington Biochemical Co.) for 12 min at 37 °C under vigourous shaking[8]. The final volume of the suspension did not exceed 2 ml. After several washings of the suspension, groups of 30 well encapsulated islets were placed in 4 ml of culture medium in Falcon Plastic Petri dishes[5]. The dishes were incubated in a humified atmosphere of 95% air and 5% CO_2 at 37 °C. Unless otherwise stated, tissue culture medium TC 199 was supplemented with 10% calf serum (Gibco: Glasgow, Scotland) previously heat inactivated (1 h, 56 °C). Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the culture medium. The basal glucose concentration was near 1 mg/ml. The islets were first cultured for 4 days to permit attachment. The medium was then replaced by fresh medium containing either low glucose (1 mg/ml), high glucose (3 mg/ml), pure pork glucagon (Novo) (10 µg/ml) or aprotinin (Novo) (10 µg/ml) for the study of insulin content and release (see results). After both the initial 4 days of culture and the 24 h experimental period, the media were recovered and frozen. The islets removed from the Petri dishes with a fine needle, were counted and transfered to 400 µl of 0.3% phosphoric acid in 60% alcohol for extraction of the insulin. In general more than 95% of the islets attached to the bottom of the Petri dish.

Determination of proteolytic activity of the medium. ^{125}I-labelled insulin and cold porcine insulin were added to the culture media in sufficient amounts to give several thousand cpm/100 µl and the required initial level of total insulin. A zero time sample was removed and frozen and the medium placed at 37 °C for up to 2 days and samples removed during the incubation and frozen. The integrity of the labelled insulin was estimated by incubating the samples at 4 °C with an excess of insulin antiserum for 4–5 h and then precipitating the antibody-bound insulin with 2.5 volumes of 96% alcohol. The ^{125}I in the supernatant and the precipitate were measured and the results expressed as % bound counts.

Immunoassay. The insulin contents of the medium and of the islets were measured by Heding's alcohol precipitation method[9] using ^{125}I-labelled porcine insulin and standards of rat insulin.

Results. 1. Degradative capacity of serum. The effect of heat treatment of serum on its ability to degrade ^{125}I-labelled insulin at 37 °C, as well as the effect of added aprotinin on the degradative capacity of serum, is shown in figure 1. Untreated serum rapidly degraded the insulin. The amount of degraded ^{125}I insulin reached 70% after 24 h of incubation. The proteolytic activity of serum was reduced by addition of aprotinin since only 40% of ^{125}I

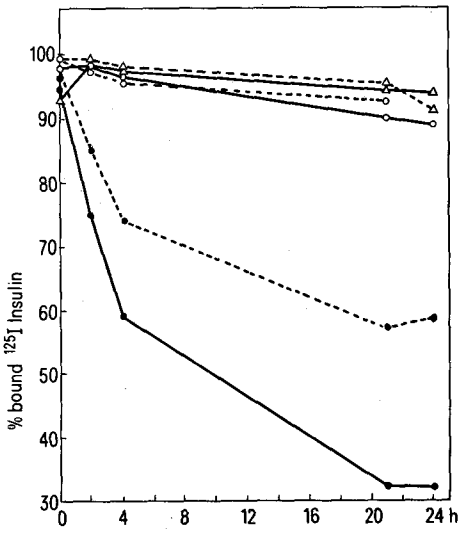

Fig. 1. Effect of heat treatment of serum on its ability to degrade ^{125}I insulin at 37 °C and effect of added aprotinin on the degradative capacity of serum. Abcissa: Incubation time of tissue culture medium TC 199 containing 10% calf serum and 1 mU/ml ^{125}I labelled pork insulin. Ordonate: Percentage of labelled insulin bound to anti-insulin serum. Untreated calf serum (●—●); heat-treated calf serum (○—○: 56 °C, 30 min; △—△: 56 °C, 1 h). Addition of aprotinin (100 µg/ml) to untreated calf serum (●---●) to heat-treated calf serum (○---○: 56 °C, 30 min; △---△: 56 °C, 1 h). Each point represents the average of 2 measurements.

insulin were degraded. When serum was heat-treated at 56 °C for 30 min or 1 h, the degradative capacity was almost abolished: Near 90% or 100% of ^{125}I insulin was recovered. Since no significant residual degradative effect of heat-treated serum could be seen, no additional aprotinin effect is possible.

2. Culture of mouse islets in medium containing normal or heat treated (1 h, 56 °C) serum. a) Analysis of the IRI content of the islets. As shown in figure 2, the content of the islets fell after the 4 days attachment phase to approximately 50% of the initial content, an observation also made by Andersson et al.[5]. The effect of glucose levels and of glucagon on the insulin content of islets cultured in both types of media was analysed during the subsequent 24 h of culture. At 1 mg/ml glucose concentration, the islets content remained quite unchanged and addition of glucagon was without effect. However, at high glucose concentration (3 mg/ml), the islets content decreased to about 1/3 of the low glucose value and glucagon nearly doubled the high glucose effect (figures 2A and B). b) Analysis of the IRI content of the media. During the 4 days attachment phase, negligible insulin was recovered from the medium containing normal serum (figure 2A), whereas little more insulin was found in the 4 days medium containing heat-treated serum (figure 2B). During the subsequent 24 h, released insulin could essentially be detected in the media containing heat-treated serum (figure 2B). At low glucose concentration, small amounts of insulin were released, even after addition of glucagon. At high glucose concentration a three-fold increase in insulin output was observed which was enhanced by glucagon ($p < 0.01$). The total insulin content (islets + medium) was significantly ($p < 0.05$) higher after 24 h stimulation than islets content at the beginning of the 24 h stimulation experiment. Glucagon added to high glucose concentration had the maximum effect.

3. Culture of islets in medium containing heat treated (56 °C, 1 h) serum and aprotinin (10 μg/ml). The protective effect of aprotinin was tested in 4 experimental series using different batches of serum. Aprotinin did not modify insulin content of media when islets were cultured during 4 or 5 days at low glucose concentration. However, addition of aprotinin favoured the recovery of insulin secreted by islets cultured at high glucose concentration with or without glucagon, but did not act on islets insulin content. Although a mean increase of 42% (23.6 to 33.5 mU/30 islets, $p < 0.01$) of insulin was obtained, the percentage varied between experiments from 25–67%. These quantitative fluctuations might be explained by a more or less residual proteolytic activity after heat treatment of the different batches of serum used.

Discussion. The present study demonstrates that normal calf serum has a degradative effect on ^{125}I insulin. The proteolytic activity in the serum can be minimized in 2 ways: a) By adding a protease inhibitor to the medium and b) by destroying the proteolytic activity by heat treatment of the serum. Both approaches have disadvantages: the addition of an inhibitor may well have unexpected side effects and the heat treatment of the serum may destroy some essential factors. It was decided that the destruction of the proteolytic factors would be more useful because one could then ensure that added polypeptides would have a reasonable persistance in the system. Heat treatment of the serum is more efficient than addition of aprotinin. The combined use of heat inactivated serum and aprotinin favoured only the recovery of insulin secreted into the culture medium after glucose or glucagon stimulation. This observation could be explained in that the protective effect of heat inactivated serum may be insufficient when high amounts of insulin were present in the culture medium. It could also reflect the presence of a more or less important residual proteolytic activity after heat treatment of various batches of serum. However, at the present time an additional effect of aprotinin on islet cell function, related to a possible hormonal contamination, cannot be excluded.

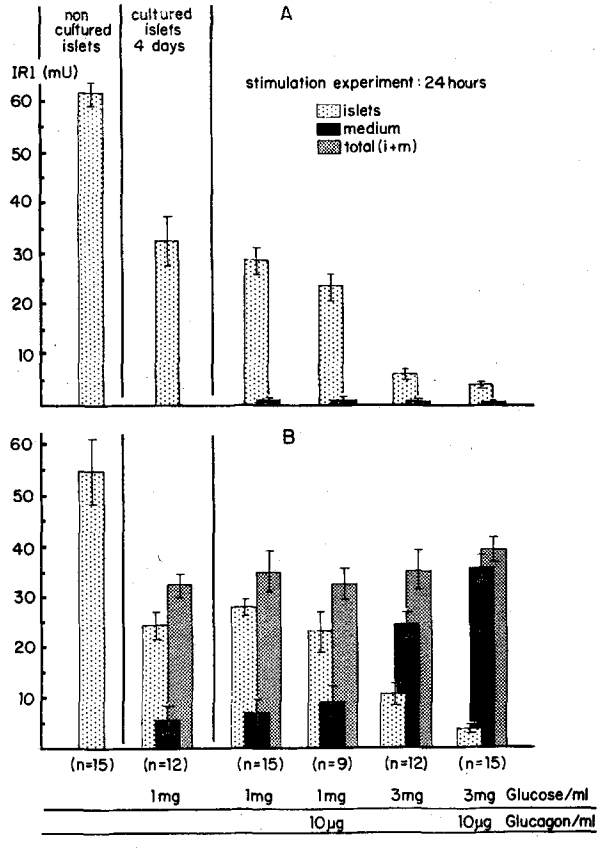

Fig. 2. Comparison between insulin content of non-cultured islets and insulin content of islets and medium after 4 and 5 days of culture. The medium contains 10%; H normal (A) or heat-inactivated calf serum (1 h, 56 °C) (B). Insulin is expressed as mU/30 islets. The numbers in parentheses represent the number of experiments. Each bar represents the mean value ± SEM.

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