Diabetologia (1984) 27: 392-396

Diabetologia

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^{125}I-glucagon-degrading activity in acid-saline extracts of rat salivary gland

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Summary. The antibody-binding ability of the glucagon-like substance in rat submaxillary gland acid saline extract was examined by affinity chromatography, and the biological activity studied using the isolated liver perfusion method. We found that the glucagon-like substances in acid saline extract could not be bound to anti-glucagon antibody and that the gel-filtration peak on ultrogel AcA 54 could increase neither glucose nor cyclic AMP output from isolated perfused rat liver. Furthermore, the radioactivity peak of ^{125}I-glucagon on Bio Gel P-6 column chromatography moved from its original position and eluted in later fractions after incubation with an acid saline extract of the submaxillary gland. In consequence, there was ^{125}I-glucagon degrading activity in the submaxillary gland, but no glucagon-related peptide. Therefore, it is suggested that the glucagon-like substance, which has been reported in acid saline extract of the rat salivary gland, may be an artifact due to tracer degrading activity.

Key words: Glucagon, salivary gland, acid saline extract, ^{125}I-glucagon-degrading activity, affinity chromatography

The existence of glucagon-like substances in the rat salivary gland has been reported in recent years [1-7]. It has been postulated that salivary gland may be one of the candidates for extrapancreatic sources of circulating glucagon, which occurs even in the pancreatectomized or eviscerated rat [1-3, 7].

However, the question whether or not the glucagon-like substance in acid saline extract of the submaxillary gland is identical to pancreatic glucagon and/or its precursor has been controversial because glucagon-like substances in the salivary gland have been found only in high molecular forms [2-5], and because of the difficulty in demonstrating it successfully by the immunohistochemical method.

Recently, Hatton et al. reported that high concentrations of glucagon-like immunoreactants measured by radioimmunoassay in brain acid saline extract were only artifacts due to ^{125}I-glucagon-degrading activity [8]. They suggested that the glucagon-like immunoreactivity in acid saline extract of the submaxillary gland likewise could be an artifact.

This investigation was carried out to examine the antibody-binding ability of the glucagon-like substance in acid saline extract by affinity chromatography, its biological activity using the isolated liver perfusion method and ^{125}I-glucagon-degrading activity by simple incubation methods. During the preparation of this paper, findings similar to these have been reported by Tahara et al. [9].

Materials and methods

Animals

Wistar rats (weight 200-250 g) were sacrificed by cutting the carotid artery and their submaxillary glands were removed and stored at $-20\,°C$ until extracted.

Extraction procedures

Several methods of extraction were used. (1) *Kenny's method* [10]: the glands were homogenized in acid alcohol by a Polytron homogenizer (Kinematica, Luzern, Switzerland) and centrifuged at 2,000 *g*. The supernatant was treated with 4.5 volumes of an alcohol-ether mixture (1.7:2.8) and the precipitate collected. (2) *Acid-ethanol method:* the glands were extracted as in method (1) but the supernatant from the centrifugation was evaporated to dryness. (3) *Boiling method:* the materials were homogenized in distilled water, boiled for 10 min, and centrifuged at 2,000 *g* for 30 min. The supernatant was used as the extract. (4) *0.2N acetic acid-boiling method:* after homogenization in 0.2N acetic acid, the same procedure was used as for method 3. (5) *2N acetic acid method:* the material was homogenized in 2N acetic acid, centrifuged at 2,000 *g* for 30 min and the supernatant fluid was collected as extract. (6) *Acid saline method:* after homogenization in saline (0.154 mol/l) acidified with HCl to pH 2.8, the sample was centrifuged at 10,000 *g* for 30 min and the supernatant was used as the extract [2-7].

Radioimmunoassay

Radioimmunoassay was performed by the method described previously [11]. The values measured by radioimmunoassay with C-terminal specific antibody 30K (Texas University, Dallas, Texas, USA) [12] or OAL123 (Otsuka Assay Laboratory, Tokushima, Japan) [13] are referred to as immunoreactive glucagon (IRG), and those measured with central portion reactive antibody K4023 (Novo, Copenhagen, Denmark) [14] or OAL196 (Otsuka Assay Laboratory, Tokushima, Japan) [15] are referred to as glucagon-like immunoreactivity (GLI) in this text. The term glucagon-like substances is used as a general term for IRG and GLI throughout the text.

Gel-filtration

The acid saline extract (0.5 ml ≡ 200 nmol/l glucagon as measured by the 30K antibody) was chromatographed on an ultrogel AcA 54 column (1.0 × 110 cm; LKB, Bromma, Sweden) in 10 mmol/l ammonium bicarbonate buffer (pH 8.6). The flow rate was approximately 6.0 ml/h and fractions of 1 ml were collected.

Liver perfusion

The biological activities of acid saline extracts were examined by liver perfusion after the method of Sugano et al. [16]. Crude acid saline extracts (≡ 0.9 nmol/l glucagon by the 30K antibody) and the gel-filtration peak (≡ 0.3 nmol/l glucagon by the 30K antibody) were perfused through rat liver for 10 min. Glucose and cyclic AMP in the effluents were measured by the glucose oxidase method and radioimmunoassay [17], respectively.

Trypsin digestion

The dried gel-filtration peak (1.5 mg protein equivalent) was dissolved in 0.1 mol/l Tris-HCl buffer (0.1 ml, pH 7.5) containing $CaCl_2$ (10 mmol/l) and trypsin (Sigma, St. Louis, Missouri, USA) at 100 μg/l. The solution was incubated at 37 °C for 1 h. After boiling for 2 min, aliquotes of enzyme-digested extracts were measured by radioimmunoassay and rechromatographed on ultrogel AcA 54 as described above.

Affinity chromatography

Rabbit anti-glucagon serum YG8, produced by ourselves, was used in this experiment. Porcine glucagon (Novo, Copenhagen, Denmark) was conjugated with bovine serum albumin (Sigma) by the glutaraldehyde method [22]. After immunization, anti-glucagon serum of low titre was obtained (final dilution used in radioimmunoassay 1:8,000). The partially purified GLI of the porcine duodenum given by Professor V. Mutt (Karolinska Institute, Stockholm, Sweden) was measured by radioimmunoassay using K4023, 30K and this antibody, YG8. The 30K immunoreactivity measured < 2.3% of the K4023 immunoreactivity in Professor Mutt's GLI, but the YG8 immunoreactivity corresponded to 36% that of antibody K4023. Consequently, the anti-glucagon serum YG8 was cross-reactive, but not completely so, with GLI.

The antiserum YG8 was purified to its γ-globulin fraction by ammonium sulphate precipitation. The purified antiserum dissolved in 0.01 mol/l Tris-HCl buffer (pH 8.4) was incubated with CN-Br-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at room temperature for 2 h and washed twice with 10 volumes of the same buffer. The coupled sepharose was put into a column (1.0 × 5.0 cm) and equilibrated with 0.01 mol/l Tris-HCl buffer (pH 8.4).

Crude acid saline extract was chromatographed on this affinity column. After incubation for 24 h, 10 volumes of 0.01 mol/l Tris-HCl buffer (pH 8.4) were eluted at a flow rate of 10 ml/h, and then five volumes of 1N acetic acid were eluted. Approximately, 1 ml fractions were collected. The pH was adjusted to 7.0–7.5 with NH_4OH and IRG was measured by radioimmunoassay with antibody 30K. Kenny's extracts of the rat hypothalamus and ^{125}I-glucagon were subjected to the same procedure as the control experiments.

^{125}I-glucagon degrading activity

Porcine crystallized glucagon (Novo, Copenhagen, Denmark) was labelled with the carrier-free solution, ^{125}I-Na (Amersham, International, Bucks, UK), using the chloramine T method [18], and the labelled ^{125}I-glucagon was purified on a QAE Sephadex A-25 (Pharmacia) column (1.0 × 30 cm), according to the method of Jorgensen and Larson [19]. ^{125}I-glucagon (~1.5 × 10^6 cpm, and approximately 45 ng) was incubated with acid saline extract (10 mg protein) of the submaxillary gland in 1.0 ml of 0.1 mol/l Tris-HCl buffer (pH 7.5) containing $CaCl_2$ (10 mmol/l) at 37 °C for 2 h and subjected to gel-filtration on Bio-Gel P-6 (1.0 × 110 cm, Bio-Rad, Richmond, California, USA). Fractions (~1 ml) were collected, and the radioactivities of these fractions were measured. As a control experiment, ^{125}I-glucagon was incubated without acid saline extact and subjected to gel-filtration.

Results

IRG and GLI concentrations in extract of the submaxillary gland

Table 1 shows IRG concentrations measured with the 30K antibody and GLI concentrations measured with antibody K4023 in rat submaxillary gland and in extracts of rat pancreas using the various extraction methods. In the extract of the submaxillary gland using Kenny's method [10], the IRG and GLI concentrations were 0.023 and 0.069 nmol/g wet weight, respectively. These values were nearly equal to those of the plasma concentration. However, in the acid-saline extract of the submaxillary gland, IRG and GLI concentrations were 1894 and 2171 nmol/g wet weight, respectively. These concentrations were similar to those in the pancreas extract obtained by Kenny's method. IRG and GLI concentrations in acid saline extract of the submaxillary gland measured with OAL123 and OAL196 were similar to those measured with 30K and K4023, respectively. It was noted that the concentration of IRG in the acid saline extract was nearly equal to the GLI concentra-

Table 1. Concentrations of glucagon-like substances in rat submaxillary gland and pancreas compared by various extraction methods

	Immunoreactive glucagon (nmol/g wet weight)	Glucagon-like immunoreactivity (nmol/g wet weight)
Submaxillary gland		
Kenny's method [10]	0.023	0.069
Acid ethanol (direct method)	0.053	0.072
Boiling method	1.36	0.169
0.2 N acetic acid boiling method	1.04	2.04
2 N acetic acid method	41.5	90.4
HCl-saline method	1894	2171
HCl-saline method	1806[a]	1158[b]
Pancreas		
Kenny's method [10]	1471	1182
HCl-saline method	25.2	20.3

[a] measured with antibody OAL123;
[b] measured with antibody OAL196

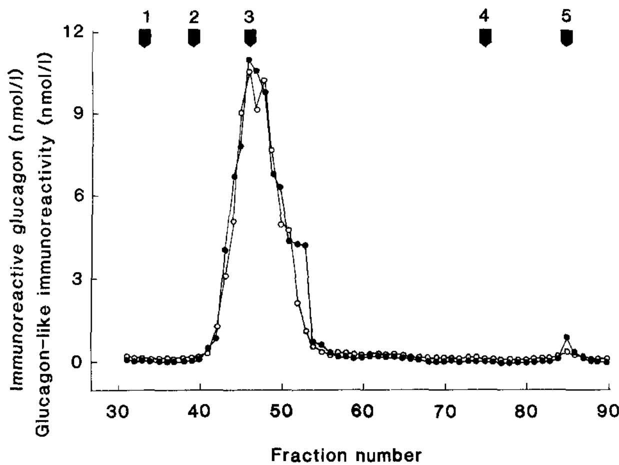

Fig. 1. Gel-filtration profile of acid saline extract of submaxillary gland on ultrogel AcA 54. ●—● immunoreactive glucagon; ○—○ glucagon-like immunoreactivity. Markers: 1: blue dextran, 2: bovine serum albumin, 3: ^{125}I-human growth hormone, 4: ^{125}I-glucagon, 5: ^{125}I-Na

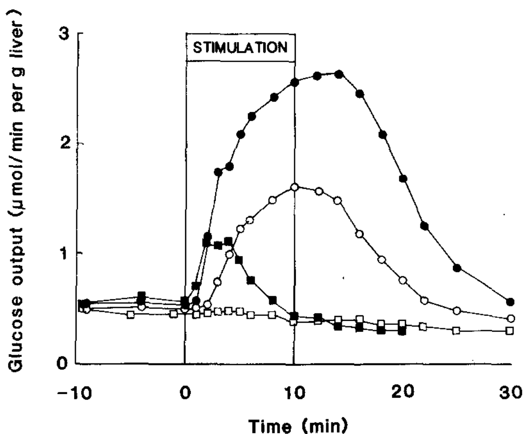

Fig. 2. Effect of acid saline extract of submaxillary gland on glucose output from isolated perfused liver. The crude acid saline extract of submaxillary gland (■—■) ($n=1$) (≡0.9 nmol/l glucagon measured by 30K antibody), the gel-filtration peak (□—□) ($n=1$) (≡0.3 nmol/l to glucagon measured by 30K antibody) as well as porcine glucagon 0.01 nmol/l (○—○) ($n=6$) and 0.1 nmol/l (●—●) ($n=4$) were perfused into isolated liver, and the glucose levels in the effluents were measured by the glucose oxidase method. Data are shown as mean values

tion. IRG and GLI concentrations in the submaxillary gland using various other extraction methods were variable and ranged between those found in Kenny's extracts and the acid saline extract.

Gel-filtration on ultrogel AcA 54

The gel-filtration profile of the acid saline extract on ultrogel AcA 54 demonstrated a single peak eluting at the position of ^{125}I-HGH (human growth hormone), which reacted identically with 30K and K4023 (Fig. 1). The molecular weight of the peak was ~20,000. There was no peak at the ^{125}I-glucagon position.

Liver perfusion

The glucose output elicited by porcine glucagon 0.01 nmol/l ($n=6$) and 0.1 nmol/l ($n=4$) increased in a dose-dependent manner in this liver perfusion experiment (Fig. 2). The crude acid saline extract from the submaxillary gland (≡0.9 nmol/l glucagon with the 30K antibody, $n=1$) gave a glucose output from the liver only one hundredth that of the immunoequivalent dose of glucagon. The pattern of glucose output raised by the crude acid saline extract was different from that raised by porcine glucagon. Furthermore, when the gel-filtration peak (≡0.3 nmol/l glucagon measured by the 30K antibody, $n=1$) was perfused, there was no effect on glucose output.

Porcine glucagon increased not only glucose but also cyclic AMP output. Cyclic AMP output was raised by porcine glucagon (0.01 and 0.1 nmol/l) to 1.41 ± 0.05 and 8.11 ± 1.96 pmol/g of liver per min (mean ± SD, $n=6$, $n=4$), respectively. However, when the crude acid saline extract and the gel-filtration peak were perfused, cyclic AMP outputs were less than the detectable level (1.27 pmol/g liver per min).

Trypsin digestion

Although IRG concentration decreased from 8.29 to 0.45 nmol/l (5.3%) after trypsin digestion, gel-filtration profiles did not alter and trypsin digestion did not elicit any new peak in the region of the lower molecular weight fraction (Figs. 1 and 3).

Affinity chromatography

Most IRG fractions in the acid saline extract of the submaxillary gland were eluted during washing procedures (Fig. 4). There was no IRG peak after lowering the pH. This phenomenon was in contrast to the affinity chromatography patterns of Kenny's extract of the rat hypothalamus and ^{125}I-glucagon in which the main IRG and radioactivity peaks were eluted after lowering the pH.

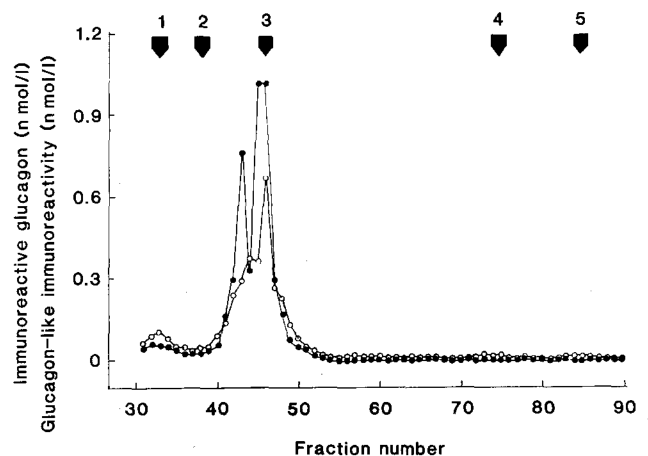

Fig. 3. Gel-filtration profile of trypsin-treated acid saline extract of submaxillary gland on ultrogel AcA 54. ●—● immunoreactive glucagon; ○—○ glucagon-like immunoreactivity. Markers as for Figure 1

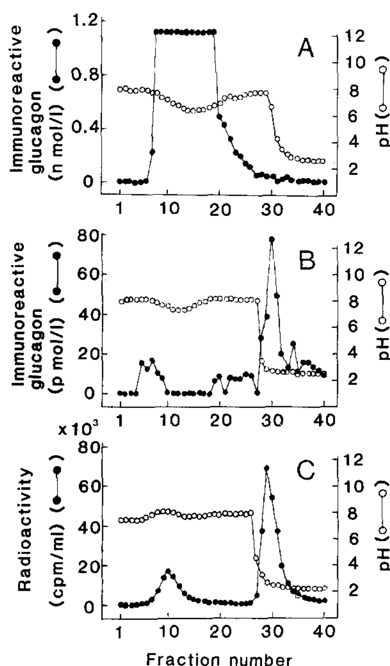

Fig. 4 A–C Affinity chromatography on sepharose 4B coupled with a rabbit anti-glucagon antiserum YG8 was used to examine the antibody-binding ability of acid saline extract of submaxillary gland (**A**), Kenny's extract of rat hypothalamus (**B**) and ^{125}I-glucagon (**C**). The pH (○—○) of each fraction (1 ml) was adjusted to 7.0–7.5 with NH_4OH and immunoreactive glucagon (IRG) concentration (●—●) was measured by radioimmunoassay with 30 K antibody. In the case of ^{125}I-glucagon, the radioactivity of each fraction was measured by a gamma-counter

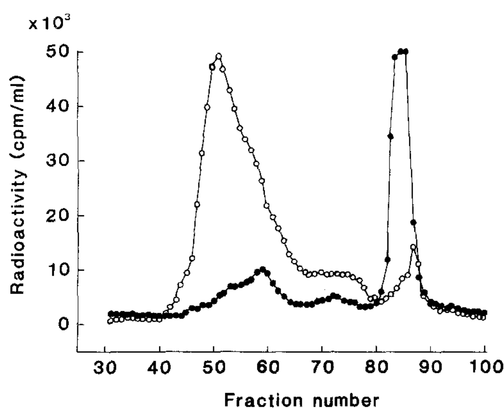

Fig. 5. Gel-filtration profile of ^{125}I-glucagon incubated with (●—●) and without (○—○) acid saline extract of submaxillary gland on Bio-Gel P-6

^{125}I-glucagon-degrading activity

When ^{125}I-glucagon was incubated with acid saline extract of the submaxillary gland, the peak of radioactivity on Bio-Gel P-6 moved from its original position to appear in later fractions (Fig. 5).

Discussion

In recent years, it has been reported that there are considerable amounts of glucagon-like substances in salivary gland extract [1–7]. Among these investigations there is a consensus that the content of glucagon-like substances is greater in the extracts from the submaxillary gland than in those from other salivary glands. Following these observations, we examined the rat submaxillary gland. It is also recognized that there are apparently high concentrations of glucagon-like immunoreactivity in acid saline extracts compared to those in acid ethanol extracts. In agreement, these studies confirm the presence of high concentrations of IRG and GLI in acid saline extracts of the submaxillary glands. The acid saline extraction method was developed originally for the extraction of higher molecular weight components which are not extractable by the traditional acid-ethanol method of Kenny [3]. This probably forms the basis of the controversy concerning the question whether glucagon-like substances in acid saline extract are identical to glucagon-related peptides including pancreatic glucagon and/or its precursors. Hatton et al. [8] raised the point that acid saline extract contain tracer degrading activity, and suggested the apparently measurable glucagon-like material could be an artifact. Perez-Castillo and Blazques [6, 7] have shown already that glucagon degradation was 67% with the acid saline extraction procedure, and had examined acid ethanol extracts.

The findings reported here that glucagon-like substances in acid saline extract of the rat submaxillary gland could not be bound to anti-glucagon antibody by affinity chromatography and that there was ^{125}I-glucagon-degrading activity in acid saline extract supports Hatton's suggestion. The glucagon-like substance found in the acid saline extract of the submaxillary gland is indeed an artifact due to tracer degrading activity.

The gel-filtration profile of acid saline extract of the submaxillary gland revealed a single high molecular weight component only. Although Lawrence et al. [2, 3] reported the molecular weight as 90,000 and Bhathena et al. [4] and Smith et al. [5] reported it as 29,000, there is at least concordance that it is a single peak of high molecular weight. Considering the high molecular weight and similarity of IRG and GLI, the putative peptide may be a large molecular precursor of which the C-terminal peptide may be identical to the C-terminal residues of pancreatic glucagon. However, glucagon precursors studied in the gut and pancreas were reported to contain C-terminal extension peptides which mask the reactivity with C-terminal specific antibody [20, 21]. This contradiction can be easily resolved if the glucagon-like substance measured by radioimmunoassay is due to tracer degrading activity. If the degrading activities are the same in both the IRG and GLI assay systems, the same values should be shown on the standard curve. Therefore, there is no relationship between the values obtained and the specific reactivity of the antibody used to the portions of the peptide sequence. Indeed, as reported here, the values of glucagon-like substances measured with four different antibodies (30K, K4023, OAL123, OAL196) were almost identical.

Hatton et al. [8] reported that stepwise dilution of acid saline extracts of the brain were parallel to the apparent immunoreactivity, but this may be erroneous. Affinity chromatography clearly identified the antibody-binding ability.

The discrepancy in apparent concentrations of glucagon-like substance using various extraction methods may depend on the amount of denaturation caused by the various extraction procedures; ethanol and boiling may accelerate the denaturation. Since trypsin digestion of acid saline extracts did not elicit any new lower molecular weight components and the gel-chromatography profile of acid saline extracts did not change before or after trypsin digestion, the apparent immunoreactivity may be due to the tracer degrading activity, which could be a protein capable of degradation by trypsin.

Difficulty in immunohistochemical demonstration of glucagon positive cells in the salivary gland has been thought curious for a long time. Only one unconfirmed report has mentioned the positive immunofluorescence of glucagon [4]. However, as substances in the salivary gland are now believed to degrade ^{125}I-glucagon but not glucagon related peptides, this accounts for failure of immunohistochemical detection.

There is a report that the biological activity of the acid saline extract was examined by displacement of ^{125}I-glucagon from rat liver plasma membrane [5]. However, if ^{125}I-glucagon can be degraded by acid saline extract, the apparent radioactivity bound to the plasma membrane would be decreased and the report's conclusion may be erroneous. One report described glycogenolytic activity in the crude extract of rat salivary gland [4]. We found also that the crude acid saline extracts increased glucose, but not cyclic AMP output. However, the finding that the gel-filtration peak of the acid saline extract did not reveal any increase in glucose output should negate this possibility. It can be argued that the effect of crude extract on glucose output may be due to other components, such as adrenaline.

On the other hand, even if the above considerations should deny the presence of glucagon-like substances in acid saline extracts of the submaxillay gland, the possibility of their presence in acid ethanol extracts may remain. However, since, as shown here and as mentioned by other authors [2–5], the values in the extracts using Kenny's methods were extremely low, the possible production of glucagon-like substances is negligible.

In conclusion, it is unlikely that the salivary gland is a source of circulating extrapancreatic glucagon.

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Received: 31 August 1983
and in revised form: 13 June 1984

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