Production of Anti-Glucagon Sera with A C-Terminal Fragment of Pancreatic Glucagon

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Synopsis

The C-terminal region-specific anti-glucagon sera were raised in rabbits using as immunogen, a conjugate of BSA and a C-terminal fragment of pancreatic glucagon. The hapten was prepared by trypsin digestion of the glucagon, which was proved to be a 1:3 mixture of glucagon (18-29) and (19-29). Six rabbits were immunized by subcutaneous injection of an emulsion of the conjugate with complete Freund's adjuvant and five of the rabbits produced antibodies to the glucagon (GC-1, GC-2, GC-3, GC-5 and GC-6). For comparison, rabbit antisera were also produced against glucagon polymer (GA-10) and syrupy glucagon fibrils (PGA-2). All these antisera as well as the pancreatic glucagon-specific antiserum 30 K were characterized with dog gut-extract (gut-GLI) and glucagon-related peptide fragments in the radioimmunoassay systems. The assay systems utilized ¹²⁵I-monosubstituted pancreatic glucagon as tracer and human mono-component glucagon as standard. All sera of the GC-series crossreacted with the dog gut-extract very weakly and antisera GC-5 and GC-6 exhibited the lowest crossreactivities with the extract, which were shown to be as low as that of 30 k. Characterization of the antiserum GC-5 with purified glucagon-related fragments indicated that the major antigenic determinant located exactly in the C-terminal region of glucagon. The present results clearly showed high efficiency of the use of the glucagon C-terminal fragment as haptenic immunogen in obtaining the C-terminal region-specific, i.e., pancreatic glucagon-specific antisera.

Since Unger *et al.* (1959) reported the successful production of glucagon antisera and development of a radioimmunoassay specific for pancreatic glucagon, a number of investigators have been engaged in the radioimmunological measurement of glucagon. However, the existence of gut glucagon-like immunoreactivity (gut-GLI) (Sutherland and deDuve, 1964; Makman and Sutherland, 1964) has often rendered difficulty in the specific measurement of pancreatic glucagon, as most of the antisera elicited against pancreatic glucagon ex-

hibited crossreactivity with gut-GLI as well. The antisera obtained by Unger *et al.* (1968), 30K and G58, and those produced by Heding (1969), K40, K47 and K964, were proved to be highly specific for pancreatic glucagon and have been widely used for its immunological measurement. However, elicitation of the pancreatic glucagon-specific antisera seemed to be by chance.

Assan and Slusher (1972) found that the structure requirements for binding with the pancreatic glucagon-specific antiserum K47 were satisfied with a C-terminal region of the glucagon and not with the N-terminal and/or central portions which were shown

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to be responsible for binding with non-specific antiserum PVP-8. A similar observation was reported by Vinik and Hardcastle (1974).

This paper described the preparation of pancreatic glucagon-specific antisera using a C-terminal fragment of glucagon obtained by tryptic digestion of bovine/porcine glucagon and characterization of the antisera.

Materials and Methods

Preparation of glucagon fragments

Trypsin digestion: Trypsin (bovine/porcine, X2 crystallized, containing 0.5% chymotryptic activity, Sigma Chem. Co.) was treated with L-1-tosylamide-2-phenylethylchloromethyl ketone TPCCK, Sigma Chem. Co.) by the method of Wang *et al.* (1965). Trypsin digestion of glucagon was carried out by a slight modification of the method by Bromer *et al.* (1957). Crystalline bovine/porcine glucagon (Sigma Chem. Co.) (100 mg) was dissolved in 0.1 N NaOH (2 ml) and pH of the solution was adjusted to 9.0 with 1 M acetic acid. To the solution was added 0.1 M triethylamine bicarbonate buffer (pH 9.0) (35 ml), which was used as substrate solution (0.27% glucagon). The TPCCK-treated trypsin (5 mg) in 0.01 N HCl (2 ml) was combined with the above substrate solution and the mixture was incubated at 30°C for 4 hr, when the reaction was terminated by acidifying the mixture to pH 2.0 with conc. HCl and the enzyme was inactivated by heating the solution in a boiling-water bath at 100°C for 5 min. The solution was then lyophilized.

Fractionation of tryptic digest: The above lyophilized digest was treated with 0.1 M citrate-HCl buffer (pH 4.0) (10 ml) and the suspension was centrifuged. The supernatant was lyophilized (60 mg), which was designated as pH 4-soluble fraction. The sediment was washed five times with the same buffer and dissolved in 0.1 M triethylamine-bicarbonate buffer (pH 10.0) (10 ml). The insoluble material was removed by centrifugation and the solution was adjusted to pH 4.0 with 0.1 N HCl. The precipitate was collected by centrifugation, washed with H₂O and dried (22 mg), which was designated as pH 4-insoluble fraction.

The pH 4-soluble fraction (60 mg) was dissolved in 0.1 M pyridine-formic acid buffer (pH 3.1) (2 ml) and the solution was applied to a column of Dowex 50×2 (200–400 mesh) (1×45 cm), which had previously been equilibrated with the same buffer. The column was eluted at a rate of 20 ml/hr with a linear gradient of pyridine-acetic acid buffer using its 0.1 M (pH 3.1) (300 ml) and 2 M (pH 5.5) (300 ml)

solutions. Fractions (6 ml) each were collected and each fraction was examined with fluorescamine reagent (Boehlen *et al.*, 1973). The fractions corresponding to the major two peaks (peak I: tube Nos. 135–145 and peak II: tube Nos. 175–185) were desalted by gel filtration on Bio-Gel P-2 and lyophilized (peak I: 11 mg and peak II: 25 mg).

The pH 4-insoluble fraction (20 mg) was dissolved in 0.03 M borate buffer (pH 9.2) (2 ml) and the solution was applied to a column of DEAE-Sephadex A-25 (1×35 cm) which had previously been equilibrated with the same buffer. The column was eluted with a 0–1.0 M linear gradient salt concentration in the same buffer using the borate buffer (200 ml) and 1.0 M NaCl in the buffer (200 ml). Elution rate was 10 ml/hr. Fractions (3 ml each) were collected and monitored by UV absorption at 276 nm. Salt concentration in each fraction was determined by titrating an aliquot of each fraction with AgNO₃. The fractions corresponding to the major two peaks (peak III: tube Nos. 22–26 and peak IV: tube Nos. 31–36) were collected, desalted by gel filtration on Bio-Gel p-2 and lyophilized (peak III: 3.2 mg and peak IV: 9.5 mg).

The pH 4-insoluble fraction and the four purified tryptic peptides corresponding to peaks I, II, III and IV were characterized by dansyl end group analysis (Gray and Hartley, 1963a and b) and by amino acid analysis of their acid hydrolysates.

Preparation of immunogens

The pH 4-insoluble fraction-BSA conjugate was used as immunogen. The pH 4-insoluble fraction (30 mg) and BSA (crystalline, Sigma Chem. Co.) (100 mg) were dissolved in 0.1 N NaOH (1 ml) and 0.1 M borate buffer (pH 9.0) (9 ml) was added. To the solution, 0.1 M glutaraldehyde (Wako Pure Chem. Co.) (3 ml) was added dropwise by constant stirring, while the pH of the solution was kept at approximately 9.0 with 0.1 N NaOH. The mixture was stirred at room temperature for 3 hr and then dialyzed against saline at 4°C for 24 hr. The yield of the conjugation was estimated by gel filtration of an aliquot of the reaction mixture on Sephadex G75 using 1% SDS containing 0.1 M NaCl as eluent. The yield of the conjugate was about 80% as calculated from the UV absorption of the eluates and the molar ratio of the peptide to BSA in the conjugate was assumed to be 8:1.

For comparative study, glucagon polymer (Heding, 1969) and syrupy glucagon fibrils (Heding, 1972) were also prepared as immunogens. To a solution of crystalline bovine/porcine glucagon (75 mg) in 0.1 N NaOH (1 ml), 0.1 M borate buffer (pH 9.5) (19 ml) was added, and then 0.4 M glutaraldehyde (5 ml) was added dropwise by stirring over a period of 1.5 hr, while the pH of the solution was kept at approximately 9.0 with 0.1 N NaOH. The reaction was terminated by adding glacial acetic acid and the

solution was submitted to dialysis against 0.9% NaCl solution and lyophilized to give glucagon polymer (60 mg). For preparation of syrupy glucagon fibrils, crystalline bovine/porcine glucagon (200 mg) was dissolved in 0.01 N HCl (2 ml) and the solution was kept at 4°C for 24 hr and centrifuged. The precipitate was washed twice with 0.01 N HCl and twice with saline and dried (60 mg).

Immunization

Six rabbits were immunized by subcutaneously injecting an emulsion with complete Freund's adjuvant of the pH 4-insoluble fraction-BSA conjugate (0.5–1.0 mg of the insoluble fraction equivalent for each animal) at 3-week intervals for 6 weeks and at a-month intervals for the following 5–10 months. Blood was obtained 10 days after the final injection. Similarly, the glucagon polymer and glucagon fibrils were injected, after being emulsified with complete Freund's adjuvant, in foot pads of rabbits by following the same schedule as above. Fifteen rabbits were immunized with the polymer and ten with the fibrils.

Preparation of ^{125}I -labelled glucagon

Glucagon was iodinated with ^{125}I Na and chloramine T in the presence of dimethylsulfoxide (DMSO) according to the procedure of Shima *et al.* (1975). Purification of the iodinated product was carried out by ion-exchange chromatography on QAE-Sephadex A-25 column according to a modification of the method of Jørgensen and Larsen (1972).

Crystalline bovine/porcine glucagon (3 μg) in 0.01N HCl (10 μl) was mixed with 0.4 M phosphate buffer (PB) (pH 7.2) (20 μl) and DMSO (5 μl). To the mixture were added successively ^{125}I Na (New England Nuclear) (1 mCi) and chloramine T (70 μg) in 0.04 M PB (pH 7.4) (20 μl). The mixture was stirred for 15–20 sec, when the reaction was terminated by adding sodium metabisulfite (120 μg) in 0.04 M PB (pH 7.4) (50 μl). The reaction mixture was combined with 2 M Tris solution (5 μl) and Tris-HCl buffer comprising 0.08 M Tris, 0.02 N HCl and 0.08 M NaCl (pH 8.6) (1 ml). An aliquot (1 ml) of the mixture was applied to a column (1.1 $\times$ 28 cm) of QAE-Sephadex A-25 which had previously been equilibrated with the Tris-HCl buffer. The column was then eluted with the buffer at a flow rate of 3 ml/hr and fractions (2 ml each) were collected. The largest peak (tube Nos. 39–49) eluting in the middle of two small peaks was proved to be monoradioiodo-glucagon which corresponded to the "Preparation A" described by Jørgensen and Larsen (1972). Specific activity of fraction No. 49 was 565 $\mu\text{Ci}/\mu\text{g}$ and fractions Nos. 49 and 50 were used in the present study.

Radioimmunoassay procedure

The standard glucagon used was human mono-component glucagon (Novo Research Institute) and the standard diluent was 0.2 M glycine buffer (pH 8.8) containing 0.25% human albumin and 1% normal sheep serum. In each incubation tube, were mixed properly-diluted anti-glucagon serum (200 μl), standard glucagon or unknown sample (200 μl), labelled glucagon (200 μl , ca. 10,000 cpm) and standard diluent (200 μl). The mixture was incubated for 48 hr at 4°C, when dextran-coated charcoal (0.5 ml) was added. After 1 hr at 4°C, the suspension was centrifuged at 3,000 rpm for 15 min at 4°C and both the supernatant and precipitate were counted for 2 min.

Preparation of dog gut-extract

Dog gut-extract was prepared according to the methods of Kenny (1955) and Valverde *et al.* (1970). Dogs were subjected to laparotomy under Nembutal anesthesia and the intestines were removed immediately after their death from Nembutal overdosage. Mucosal scraps were obtained from the jejunum and they were extracted by the acid-alcohol method (Kenny, 1955). The product from the last-step dialysis was subjected to gel filtration on Bio-Gel p-10 using 0.05 M NH_4HCO_3 as eluent (Valverde *et al.*, 1970). The eluates, so-called "peak I", which were located before ^{125}I -insulin marker and exhibited immunoreactivity to antiserum GA-10 (described in RESULTS AND DISCUSSION section) were pooled and lyophilized.

Table 1. Amino acid composition of pH 4-insoluble fraction

Amino acid	Recovered umoles per mg of peptide	Molar ratios (Ala assumed as 1.00)	Theoretical ratios in PG (18–29)
Lys	0.011	0.02	—
His	0.005	0.01	—
Arg	0.159	0.34	1
Asp	1.098	2.31	2
Thr	0.525	1.11	1
Ser	ND	ND	—
Glu	1.064	2.24	2
Gly	0.020	0.04	—
Ala	0.475	1.00	1
Val	0.470	0.99	1
Met	0.328	0.70	1
Leu	0.465	0.98	1
Tyr	0.015	0.03	—
Phe	0.379	0.80	1

Results and Discussion

Tryptic peptides of pancreatic glucagon

Table 1 shows amino acid ratios in an acid hydrolysate of the pH 4-insoluble fraction from the tryptic digest of glucagon. The dansyl end group analysis of the fraction indicated the presence of two major peptides that were terminated with Ala and Arg residues at the N-termini, respectively. Bromer *et al.* (1957) previously reported that trypsin digestion of glucagon yielded four major peptide fragments cor-

responding to the sequences 1-12, 13-17, 18-29 and 19-29 which were called N, M, C₁ and C₂ fragments, respectively. Since Arg residue is in position 18 and Ala in position 19, the present results of the amino acid analysis and dansyl end group analysis indicate that the pH 4-insoluble fraction consists mainly of glucagon 18-29 and 19-29 fragments corresponding to C₁ and C₂ in a ratio of approximately 1 to 3. This material was satisfactorily used as haptenic antigen for production of anti-glucagon antisera.

The pH 4-soluble and -insoluble fractions

Fig. 1. Chromatographic purification of (a) the pH 4-soluble fraction on Dowex 50x2 and (b) the pH 4-insoluble fraction on DEAE-Sephadex A-25.

from the tryptic digest of glucagon were further purified by ion exchange chromatography on Dowex 50 column of the pH 4-soluble fraction is shown in Fig. 1a. The chromatographic fractionation of the pH 4-insoluble fraction on DEAE-Sephadex column is shown in Fig. 1b. The two major peaks, I and II, in Fig. 1a and two major peaks, III and IV, in Fig. 1b were examined by dansyl end group analysis and amino acid analysis of their acid hydrolysates (Table 2). The analytical data proved that the peaks I, II, III and IV are glucagon 1-12, 13-17, 18-29 and 19-29 fragments, respectively, and that these purified tryptic

peptides are able to be used safely as substrates for characterization of the glucagon antisera.

Production of antisera

Production of antisera was attempted in rabbits using as immunogens, the pH 4-insoluble fraction—BSA conjugate, glucagon polymer and syrupy glucagon fibrils. After six injections, production of anti-glucagon sera was detected in five rabbits given the conjugate (GC-1, 2, 3, 5 and 6), one given the polymer (PGA-2) and one given the fibrils (GA-10). Table 3 indicates the affinity constants (equilibrium

Table 2. Amino acid compositions of chromatographically purified four preparations from pH 4-soluble and -insoluble fractions

Amino acid	Peak I		Peak II		Peak III		Peak IV	
	Molar ratios		Molar ratios		Molar ratios		Molar ratios	
	Found	Expected in PG (1-12)	Found	Expected in PG (13-17)	Found	Expected in PG (18-29)	Found	Expected in PG (19-29)
Lys	1.05	1	0.94	1	—	0	—	0
His	0.99	1	—	0	—	0	—	0
Arg	—	0	—	0	1.17	1	—	0
Asp	1.00	1	1.01	1	2.12	2	2.09	2
Thr	1.90	2	—	0	0.91	1	0.96	1
Ser	3.29	3	0.93	1	0.08	0	—	0
Glu	1.01	1	—	0	2.09	2	2.00	2
Gly	1.00*	1	—	0	0.09	0	—	0
Ala	—	0	—	0	1.00*	1	1.00*	1
Val	—	0	—	0	1.13	1	1.01	1
Met	—	0	—	0	0.90	1	0.96	1
Leu	—	0	1.00*	1	0.94	1	1.00	1
Tyr	0.97	1	0.82	1	—	0	—	0
Phe	0.98	1	—	0	1.03	1	1.15	1

* Assumed as 1.00

Table 3. Physical characterization of anti-glucagon antisera

Antiserum	Affinity constant		Number of binding site	
	high	low	low	high
	$\times 10^{10} \text{ M}^{-1}$	$\times 10^9 \text{ M}^{-1}$	$\times 10^{-14} \text{ mol/ml}$	$\times 10^{-14} \text{ mol/ml}$
GC-1	6.3	8.3	2.9	9.3
GC-2	2.9	5.6	7.3	24.4
GC-3	1.9	8.3	7.7	10.4
GC-5	14.3	—	1.3	—
GC-6	5.3	13.7	2.3	4.6
GA-10	1.1	—	8.6	—
PGA-2	7.7	14.7	1.1	2.5

Fig. 2. Crossreactivities of dog gut-extract (○) in radioimmunoassay systems using antisera (a) GA-10 (1:10,000), (b) PGA-2 (1:11,000), (c) GC-1 (1:150,000), (d) GC-2 (1:50,000) (e) GC-3 (1:60,000), (f) GC-5 (1:160,000), (g) GC-6 (1:50,000) and (h) 30 K (1:50,000). Labelled antigen: ¹²⁵I-pancreatic glucagon and standard: human mono-component glucagon (●). The dilution scale for dog gut-extract is fixed in such way that the dose-response curve of the extract is superimposed on that of the standard glucagon in the GA-10 assay system.

constants) and number of the binding sites to pancreatic glucagon of these seven antisera. All of the antisera exhibited affinities to the glucagon sufficiently high enough to develop sensitive radioimmunoassay for glucagon.

Characterization of antisera

Crossreactivities of antisera with dog gut-extract: Using pancreatic mono-component glucagon as standard and ^{125}I -glucagon (monoradioiodo form) as tracer, radioimmunoassays were developed with the seven antisera obtained and crossreactivities of the antisera with dog gut-extract (gut-GLI) were examined in the systems. Figs. 2a—2g show the results of the assays. Crossreactivity with the dog gut-extract was also tested, for comparison, in the system using pancreatic glucagon-specific antiserum 30K (Fig. 2h). Antiserum GA-10 exhibited the highest crossreactivity with the extract and its dilution curve was completely superimposable on the dose-response curve of the standard pancreatic glucagon as shown in Fig. 2a. As compared with GA-10, the other six antisera as well as 30K were less reactive with the gut-extract. Relative crossreactivities of the antisera to the extract are listed in Table 4. The crossreactivity of the present extract in the 30K system was comparable to that of the dog gut-extract measured by Eisentraut *et al.* (1968) in their 30K assay system. The antisera of GC-series showed relative crossreactivities ranging from 0.64 to 1.5 % of that of GA-10, which seemed to be satisfactorily low in order to discriminate gut-GLI from pancreatic glucagon. From these results, it may be concluded that protein conjugate of the C-terminal fragment of glucagon is an efficient immunogen for production of antisera specific to pancreatic glucagon.

Crossreactivities of antisera with glucagon-related peptides: Among the five antisera of GC-series, GC-5 showed a linear

Scatchard plot and the highest sensitivity in the radioimmunoassay and it could recognize gut-GLI to the least extent. Thus, crossreactivities of glucagon-related peptides were examined in the system with GC-5 in comparison with those in the systems using GA-10, PGA-2 and 30K. The glucagon-related peptides used were pancreatic glucagon (PG) and its fragments, PG(1–12), PG(13–17), PG(18–29) and PG(19–29). The inhibition curves of these peptides in each system are shown in Figs. 3a—3d. And relative crossreactivities of these peptide fragments in the four systems are summarized in Table 5, which were calculated from the amount of a fragment required for 50 % inhibition of ^{125}I -glucagon binding. The non-specific antiserum GA-10 crossreacted with none of the peptide fragments, while relatively high crossreactivities of PGA-2 were observed with the C-terminal peptides PG(18–29) and PG(19–29) rather than with the N-terminal fragment PG(1–12). Crossreactivity of specific-antiserum 30K with PG(18–29) was 48 % of that with pancreatic glucagon and the reactivity with PG(19–29) was decreased to approximately half of with that the (18–29) fragment,

Table 4. Crossreactivity of dog gut-extract with various anti-glucagon antisera

Antiserum	Crossreactivity of dog gut-extract* (ng/ml pancreatic glucagon equivalent)	Relative crossreactivity (%)
GA-10	44.00	Assumed as 100
PGA-2	3.40	7.70
30 K	0.29	0.64
GC-1	0.36	0.82
GC-2	0.48	1.09
GC-3	0.66	1.50
GC-5	0.29	0.64
GC-6	0.26	0.59

* A solution containing dog gut-extract equivalent to 44.00 ng standard pancreatic glucagon per ml as determined in the GA-10 system was assayed in each system, and the crossreactivity of the extract in each system was expressed by equivalent weight of the standard glucagon.

indicating an important role of the Arg residue in position 18 in interaction of the antigen with antiserum 30K. This antiserum reacted negligibly with PG(1-12) and

PG(13-17). These results with 30K are in well agreement with the data reported by Faloona and Unger (1974). Antiserum GC-5 crossreacted with PG(18-29) and

Fig. 3. Inhibition curves of glucagon-related peptides in radioimmunoassay systems using antisera (a) GC-5, (b) GA-10, (c) PGA-2 and (d) 30 K. Labelled antigen: ^{125}I -pancreatic glucagon and peptides: pancreatic glucagon (PG) (●), PG (18-29) (○), PG (19-29) (▲), PG (13-17) (□) and PG (1-12) (■).

Table 5. Relative crossreactivities of pancreatic glucagon-related peptides in glucagon radioimmunoassay systems using four different antisera

Antiserum	Pancreatic glucagon PG (1-29)	PG (18-29)	PG (19-29)	PG (13-17)	PG (1-12)
GC-5	1	1	1	0.002	<0.001
30 K	1	0.48	0.22	<0.001	<0.001
PGA-2	1	0.23	0.15	<0.001	<0.001
GA-10	1	<0.01	<0.01	<0.001	<0.001

Relative crossreactivity of a peptide was calculated by dividing the dose of the standard glucagon required for 50% inhibition by that of the corresponding peptide.

PG(19-29) to the same extent as with pancreatic glucagon. Very little but apparent crossreactivities of GC-5 with PG (1-12) and PG (13-17) may be due to trace the contamination of glucagon or/and the N-terminal fragment(s) in the pH 4-insoluble fraction that had been used as haptenic antigen. It has become, thus, clear that the C-terminal region-specific antiserum GC-5 obtained in this study can almost specifically react with pancreatic glucagon and the present results have provided an additional support to the proposal that antibodies specific for pancreatic glucagon interact with the hydrophobic C-terminal region in the molecule (Assan and Slusher, 1972).

Utilizing the pancreatic glucagon-specific antiserum GC-5, the study on practical radio-immunological measurement of glucagon in biological fluids is now under way.

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References

- Assan, R., and N. Slusher (1972). *Diabetes* 21, 847.
- Boehlen, P., S. Stein, W. Dairman and S. Udenfriend (1973). *Arch. Biochem. Biophys.* 155, 213.
- Bromer, W. W., A. Staub, L. G. Sinn and O. K. Behrens (1957). *J. Amer. Chem. Soc.* 79, 2801.
- Eisentraut, A. M., A. Ohneda, E. Parada and R. H. Unger (1968). *Diabetes* 17 (Suppl. 1), 321.
- Faloona, G. R., and R. H. Unger (1974). *Israel J. Med. Sci.* 10, 1324.
- Gray, W. R., and B. S. Hartley (1963a). *Biochem. J.* 89, 59.
- Gray, W. R., and B. S. Hartley (1963b). *ibid.* 89, 379.
- Heding, L. G. (1969). *Horm. Metab. Res.* 1, 87.
- Heding, L. G. (1972). *Glucagon* (edited by P. J. Lefebvre and R. H. Unger) Pergamon Press, Oxford, p. 187.
- Jørgensen, K. H., and U. D. Larsen (1972). *Horm. Metab. Res.* 4, 223.
- Kenny, J. (1955). *J. Clin. Endocrinol. Metab.* 15, 865.
- Makman M. H., and E. W. Sutherland (1964). *Endocrinology* 75, 127.
- Shima, K., N. Sawazaki, R. Tanaka, S. Tarui and M. Nishikawa (1975). *ibid.* 96, 1254.
- Sutherland, E. W., and C. deDube (1964). *J. Biol. Chem.* 175, 663.
- Unger, R. H., A. M. Eisentraut, M. S. McCall, S. Keller, H. C. Lanz and L. L. Madison (1959). *Proc. Soc. Exp. Biol. Med.* 102, 621.
- Unger, R. H., A. Ohneda, I. Valverde, A. M. Eisentraut and J. Exon (1968). *J. Clin. Invest.* 47, 48.
- Valverde, I., D. Rigopoulou, J. Marco, G. R. Faloona and R. H. Unger (1970). *Diabetes* 19, 614.
- Vinik, A. I., and A. Hardcastle (1974). *Horm. Metab. Res.* 6, 396.
- Wang, S. S., and F. H. Carpenter (1965). *J. Biol. Chem.* 240, 1619.