J. Membrane Biol. 20, 301–318 (1975)
© by Springer-Verlag New York Inc. 1975

Regulation of 3′,5′-Cyclic AMP-Dependent Protein Kinase in the Plasma Membrane of Cod (*Gadus callarius*) and Mouse Islets

Brian Davis and Norman R. Lazarus

Diabetes Research Unit, The Wellcome Foundation Limited,
Temple Hill, Dartford, Kent, DA 1 5 AH, England

Received 11 March 1974; revised 16 September 1974

Summary. The membranes of both mouse and cod islets contain a protein phosphokinase enzyme. 3′,5′-cyclic AMP, ouabain, phosphoenolpyruvate and glucose-6-phosphate enhance protein phosphokinase activity. Protein kinase which is membrane bound is unable to use exogenous protein (histone) as a substrate and phosphorylates specific proteins in the membranes. These proteins have been partially characterized.

The plasma membrane of mouse pancreatic β-cells has been shown to contain adenylate cyclase. The presence of this enzyme suggests that 3′,5′-cAMP may play a role in insulin secretion. The most likely effector site of 3′,5′-cAMP is a protein phosphokinase. The purpose of this study was to investigate whether the plasma membrane of the β-cells of mouse and cod islets contained a protein phosphokinase and to determine the factors modulating the activity of this enzyme.

Materials and Methods

[γ^{32}P] ATP (ammonium salt) was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, U.K. Calf thymus histone mixture, ovalbumin, urease, bovine thyroglobulin, lysozyme, L-glucose, phosphoenolpyruvate, ouabain and diphenylhydantoin were purchased from Sigma (London) Chemical Co. Ltd. Rabbit muscle lactate dehydrogenase, pyruvate kinase and adenosine 3′,5′-monophosphate were obtained from the Boehringer Corporation (London) Ltd. Worthington crude collagenase and adenosine-5′-triphosphate were supplied by Cambrian Chemicals Ltd., Croydon, Surrey, U.K. Acrylamide was purchased from Eastman Kodak Co., Rochester, N.Y. NE 220 Scintillation Fluid was purchased from Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, U.K. Schwarz specific 3′,5′-cAMP antibody was purchased from Becton and Dickinson Ltd., Wembley, Middlesex, U.K. Theophylline was obtained from Burroughs Wellcome and Company, Dartford, Kent, U.K. and Sephadex G-200 super-

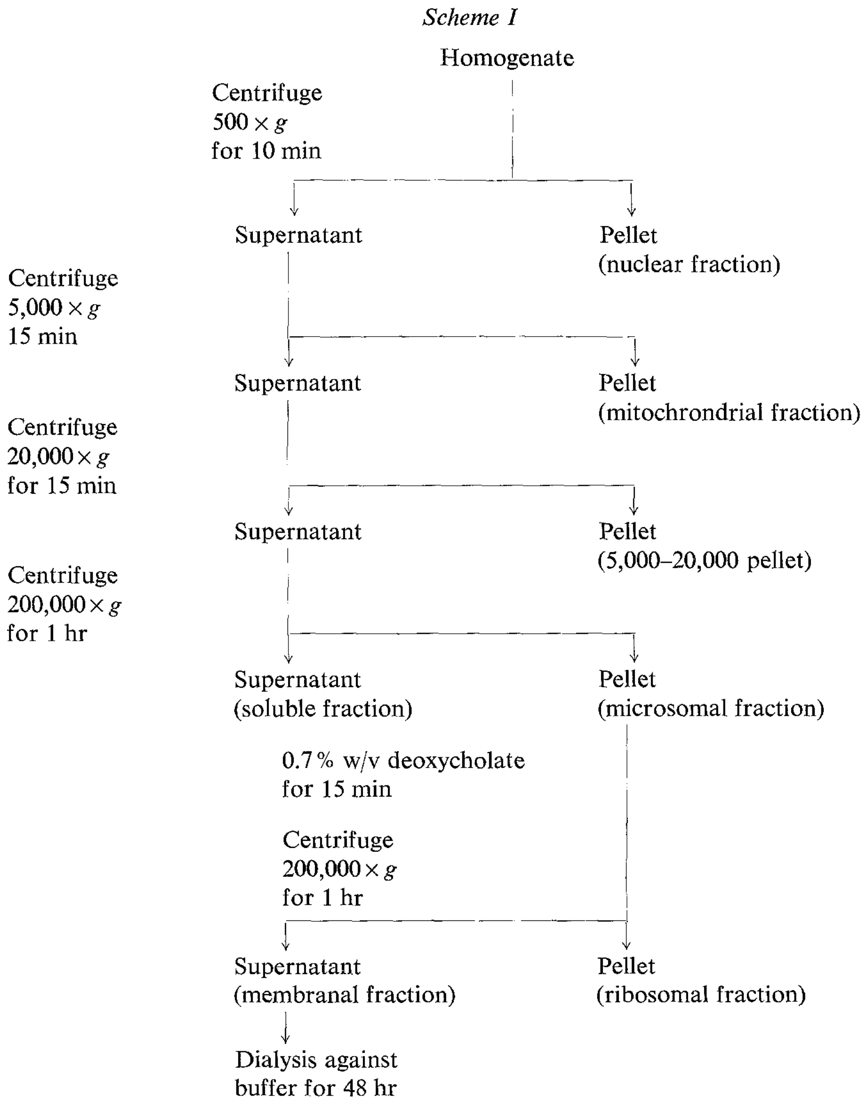
fine from Pharmacia Ltd., Ealing, London. All other chemicals used in the preparation of solutions were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Normal male albino mice, LACA Carshalton strain, were supplied by Evans Animal Supplies Ltd., Carshalton, Surrey, U.K. Frozen cod islet tissue (Brockmann bodies) was the generous gift of Dr. P. T. Grant, Director of the Institute of Marine Biochemistry, Aberdeen, U.K.

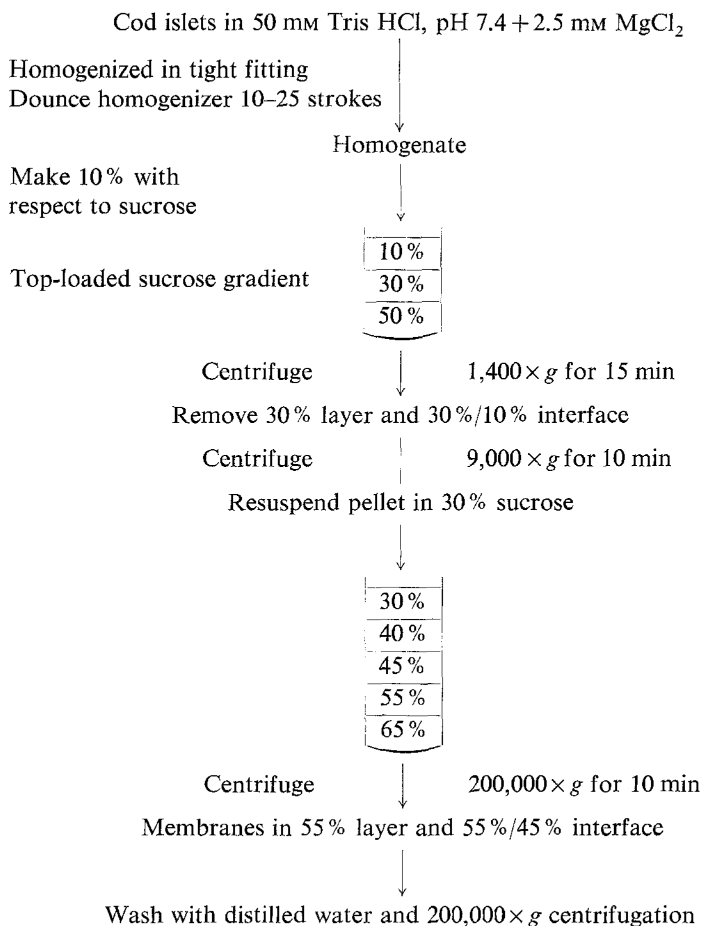
Preparation of Protein Phosphokinase

Cod: Frozen Islets. Frozen cod Brockmann bodies were chopped and homogenized in 10 vol 0.02 M tris HCl (pH 7.4) containing 0.1 M KCl, 0.005 M $MgCl_2$ and 0.006 M β-mercaptoethanol at 4 °C, using a Polytron PT 10 homogenizer at maximum for 30 sec. The homogenate was then fractionated according to Scheme I.

Scheme I



Scheme II



The deoxycholate-treated membranes, after dialysis, yielded an enzyme preparation having a threefold increases in specific activity over the crude microsomal fraction. Membranes prepared by this method are referred to as preparation I throughout the text.

Cod: Fresh Islets. Fresh cod islet tissue was also obtained and fractionated by the method of Kiehn and Holland (1970). The method shown in Scheme II yields purified plasma membranes free of any microsomal and ribosomal contamination. This preparation is referred to as preparation II or as purified plasma membranes.

Mouse. 90,000 mouse islets were collected by a modification of the collagenase technique of Howell and Taylor (1968) and Davis and Lazarus (1972). The islets were fractionated in the same way as the frozen cod tissue (preparation I).

Enzyme Markers Present in the Fractions Prepared from Frozen Cod Islets

5′-Nucleotidase. The assay of 5′-nucleotidase was performed on all fractions. The assay volume of 1 ml contained 20 mM 5′-AMP in veronal buffer at pH 9.0 and 2 mg

protein. Tubes were incubated for 15 min and the reaction terminated by the addition of 2 ml 10% w/v trichloroacetic acid. The protein was removed by centrifugation and the supernatant assayed for Pi by the method of Fiske and Subbarow (1925).

Cytochrome c. Cytochrome *c* oxidase activity was assayed by the method of Cooperstein and Lazarow (1951).

Glucose-6-Phosphatase. Fractions were assayed by the phosphate release method. 0.5 mg protein from islet fractions was incubated in 1 ml 0.2 M G-6-P in 0.1 M citrate buffer (pH 6.3), at 15 °C. After 20-min incubation 2 ml 10% w/v trichloroacetic acid was added to each tube and the samples were then centrifuged to remove protein. The supernatants were assayed for inorganic phosphorus by the method of Fiske and Subbarow (1925).

Adenylate Cyclase. Adenylate cyclase activity was assayed in preparations I and II as previously described (Davis & Lazarus, 1972).

Phosphodiesterase Activity. Phosphodiesterase activity was assayed in preparation II using the method of Bowen and Lazarus (1973).

ATPase. ATPase activity was examined in preparation I. The assay volume was 1.7 ml and contained (in mM): 100 imidazole, 2 ATP, 5 $MgCl_2$, 50 NaCl, 5 KCl all at pH 7.2. 0.5 mg membrane protein was added to each tube and the tubes were incubated at 15 °C for 5 min. The reaction was terminated by the addition of 0.5 ml 30% w/v perchloric acid. Tubes were then centrifuged and the supernatants assayed for Pi by the method of Fiske and Subbarow (1925). Na^+/K^+-dependent ATPase was estimated from the amount of inhibition produced by 5 mM ouabain.

Electron-microscopy. The specimens were fixed in buffer glutaraldehyde (pH 7.2) and post-fixed in osmium tetroxide.

Dehydration was carried out using 70% w/v ethanol and three changes of absolute ethanol. Two changes of propylene oxide were applied and infiltration and embedding continued in maraglas (Freeman & Spurlock, 1962).

"Silver" sections were cut on an LKB Ultratome, stained with 2% w/v aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) and then viewed in a Philips-200 electron-microscope.

Protein Estimation. Protein estimations were performed by the method of Lowry, Rosebrough, Farr and Randall (1951).

Assay of Protein Phosphokinase Activity. The assay was based on the rate of transfer of ^{32}P from [$\gamma^{32}P$] ATP to acid precipitable protein and was a modification of the method of Kuo, Krueger, Sanes and Greengard (1970). The assay volume was 0.2 ml and contained 1–5 µM [$\gamma^{32}P$] ATP [1.5×10^3 cpm/pmole] (5 µM for routine assays), 10 mM $MgCl_2$, 2.0 mM theophylline, 0.3 mM ethylene glycol tris (β-amino ethyl ether)-N,N′-tetraacetic acid (EGTA), 50 mM sodium glycerol phosphate buffer, pH 6.5, and 10–100 µg membrane protein. Since Weller and Rodnight (1973) showed that theophylline inhibited brain membrane-bound protein phosphokinase, the effect of individual constituents of the assay system were separately tested. Theophylline did not inhibit the enzyme but enhanced activity in the presence of 3′,5′-cAMP. EGTA protected the enzyme from inhibition by calcium ions, and was itself nonstimulatory.

The assay mixture was equilibrated at 15 °C for cod enzyme (or 30 °C for mouse enzyme) for 15 min before addition of [$\gamma^{32}P$] ATP and incubated for 5 min. The reaction was terminated by the addition of 2 ml 5% w/v trichloroacetic acid. 0.2 ml 0.63% w/v bovine albumin was added as a carrier and a further 2 ml 5% trichloroacetic acid added. Tubes were centrifuged at $1{,}000 \times g$ for 5 min and the supernatant discarded. Protein was redissolved in 0.1 ml 1 M NaOH at 20 °C, mixed and allowed to stand for 1 min.

A further 4 ml trichloroacetic acid was added. The tubes were centrifuged and the protein redissolved in 0.1 ml 1 M NaOH. A further precipitation was carried out and the redissolved protein washed into a scintillation vial with 2 × 5 ml NE 220 scintillation fluid. ^{32}P was then counted in a Nuclear Enterprises automatic scintillation counter (type NE 8312). Counts were accumulated to give a counting error of $\pm 2\%$ or less. A protein control containing either boiled membranes or bovine albumin was included in all experiments. The amount of phosphorus transferred was determined from the specific radioactivity of the substrate. All assays were performed in duplicate or triplicate and points in figures are arithmetical means.

Determination of Molecular Weight of Membrane Protein Kinase by Gel Filtration

A portion of 2 mg microsomal protein was treated with 1 ml 0.1% w/v Triton X-100 for 20 min at room temperature. The remaining insoluble material was removed by centrifugation at 200,000 × *g* for 1 hr. The supernatant was then dialyzed against 100 vol 0.005 M tris HCl buffer, pH 7.5, containing 0.002 M EDTA, for 24 hr with two changes of buffer.

A 27.5 × 0.9 cm G-200 Sephadex superfine column was equilibrated with 0.005 M tris HCl, pH 7.5, containing 0.002 M EDTA and the flow rate adjusted to 0.5 ml per hour.

500 μg of the following marker proteins were applied: thyroglobulin (680,000 mol wt), urease (480,000 mol wt), lactate dehydrogenase (140,000 mol wt), ovalbumin (44,000 mol wt) and lysozyme (14,700 mol wt). Fractions of 0.25 ml were collected and monitored for absorbance on a Pye Unicam SP 500 spectrophotometer. After calibration of the column the solubilized enzyme was applied and fractions monitored for protein phosphokinase activity and absorbance at 280 nm.

Solubilization of Membranes with 0.7% w/v Deoxycholate

The membrane components which were being phosphorylated by the protein kinase were further characterized by the following methods.

Method 1. Three mg of membrane was treated with 0.7% w/v deoxycholate and allowed to stand at room temperature for 15 min. The tube was then centrifuged at 200,000 × *g* for 1 hr. The supernatant was dialyzed against 0.005 M tris HCl buffer, pH 7.5, containing 0.002 M EDTA and 0.7% w/v deoxycholate for 2–3 hr and then applied to a 30 × 1.3 cm Sephadex G-200 superfine column and eluted with the same buffer. Fractions of 1.5 ml were collected. The fractions were assayed for protein and then dialyzed against distilled water, lyophilized and reconstituted in 50 μliters of distilled water. A portion of 25 μliters of each fraction was then assayed for protein phosphokinase substrate using purified rat muscle protein phosphokinase enzyme. By this means a substrate profile was obtained. The tubes containing the phosphorylated protein peaks were pooled, lyophilized and applied to a calibrated G-200 superfine column as described previously. From the elution patterns the apparent molecular weights of the protein peaks were determined.

Method 2. Tubes were set up containing the following constituents: 8 μmoles $MgCl_2$, 1.6 μmoles theophylline, 0.24 μmoles EGTA, 4 nmoles 3′,5′-cAMP, 4 nmoles [$\gamma^{32}P$] ATP, 40 μmoles glycerol phosphate buffer, pH 6.5, and 0.5 ml microsomal suspension in a total volume of 1 ml.

The assay mixture was incubated at 15 °C for 5 min. One milliliter of 1.4% sodium deoxycholate was then added and the contents of the tube were mixed well and then

centrifuged at 200,000 × *g* for 1 hr. The supernatant was then dialyzed against 0.005 M tris HCl buffer, pH 7.5, containing 0.002 M EDTA and 0.7 % w/v deoxycholate for 24 hr. The sample was then applied to a 30 × 1.3 cm Sephadex G-200 superfine column and eluted with the same buffer. Fractions were monitored for protein and ^{32}P.

The peaks obtained by this procedure were pooled and dialyzed against distilled water for 24 hr, lyophilized and then subjected to polyacrylamide electrophoresis.

Polyacrylamide Electrophoresis

Samples were applied to 15 % w/v acrylamide gels in silica tubes (0.6 mm i.d. 5 cm long) in a solution of 20 % w/v sucrose containing 0.1 % w/v bromophenol blue. The running buffer used was tris glycine buffer at pH 8.3 containing 0.7 % deoxycholate. A current of 1 mA per tube was applied for 15 min and then 2 mA per tube for about 2 hr. The gels were then scanned for absorbance at 280 nm on a Canalco Densitometer Model G (Canalco, Rockville, Md). The gels were then removed from the tubes, sliced into 2-mm lengths and then placed in scintillation vials containing 0.5 ml 0.1 M NH_4OH. The vials were shaken and 10 ml NE 220 scintillation fluid was added. The fractions were then monitored for ^{32}P.

Results

Definition of Plasma Membranes Obtained

Enzyme Markers

Cytochrome *c* oxidase, 5′-nucleotidase and glucose-6-phosphatase were assayed in all fractions. The activities obtained as a percentage of total homogenate activity are shown in Table 1 for both preparation I and preparation II.

Table 1. Activities of cytochrome *c* oxidase, 5′-nucleotidase and glucose-6-phosphatase in various islet fractions

Islet fraction	Activity % total homogenate		
	Cytochrome *c* oxidase	5′-Nucleotidase	Glucose-6-phosphatase
Preparation I			
Nuclear pellet	7	6	1
Mitochondrial pellet	76	—	2
5,000–20,000 × *g* pellet	1	2	52
200,000 × *g* pellet	—	72	3
200,000 × *g* supernatant	—	—	19
Deoxycholate-treated supernatant	—	71	2
Deoxycholate-treated pellet	—	1	1
Preparation II			
Purified plasma membranes	—	81	—

Adenylate cyclase was also present in preparations I and II giving basal activities of 35.5 pmoles 3′,5′-cAMP formed per min/mg protein and 61.2 pmoles 3′,5′-cAMP formed per min/mg protein, respectively. Total ATPase activity was 140 nmoles ATP hydrolyzed per min/mg protein. Na^{+}/K^{+} ATPase activity determined in the presence of 5 mM ouabain was 96.3 nmoles ATP hydrolyzed per min/mg. Phosphodiesterase was also present in preparation II having a basal activity of 961.3 nmoles 5′-AMP formed per min/mg protein. 50% inhibition was obtained with 6 mM theophylline.

Electron-microscopy

Preparation I. Electron-microscopy of preparation I showed plasma membrane contaminated with smooth and rough endoplasmic reticulum and with some cellular debris. Treatment with 0.7% deoxycholate removed a large amount of nonmembranal material (Johnson, Maeno & Greengard, 1971).

Preparation II. Examination of preparation II showed almost entirely pure plasma membrane with very little extraneous material (Fig. 1).

These results in conjunction with the enzyme marker data indicate that preparation I consisted largely of plasma membrane material while preparation II was almost entirely plasma membrane.

The deoxycholate from preparation I was removed by extensive dialysis over 48 hr. The activity of the protein phosphokinase after deoxycholate removal was identical to that found in preparation II under all experimental conditions studied.

Protein Phosphokinase Activity

The protein phosphokinase in preparation I and preparation II was stable to storage at −70 °C. The pH optimum for the cod and mouse enzyme was 6.5. The optimum temperature for cod enzyme activity was 15 °C. The mouse enzyme was assayed at 30 °C. The time course for membrane phosphorylation is shown in Fig. 2. Calcium inhibited the cod enzyme (50% inhibition at 0.5 mM Ca^{++}). The presence of EGTA removed Ca^{++} inhibition.

D-glucose at concentrations of 17, 8.5 and 4.25 mM had no effect on the activity of cod or mouse enzyme (preparation I). L-glucose at the same concentrations was used as the control.

To test the effect of 3′,5′-cAMP on the protein phosphokinase activity, the enzyme was assayed in the presence of increasing amounts of exogenous 3′,5′-cAMP in the presence and absence of fluoride, a known adenylate

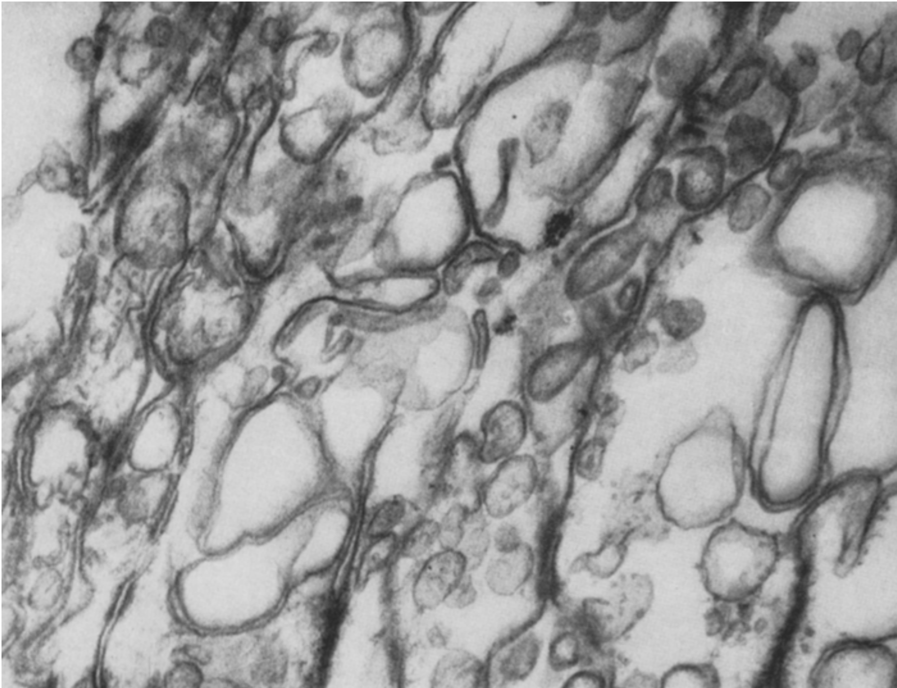


Fig. 1. Membranes from preparation II were fixed in glutaraldehyde and post-fixed in osmium tetroxide. Following dehydration they were embedded in maraglas, sections were cut and stained in uranyl acetate and lead citrate then viewed in a Philips-200 electron-microscope. The plate shows membranes at a magnification of 131,150

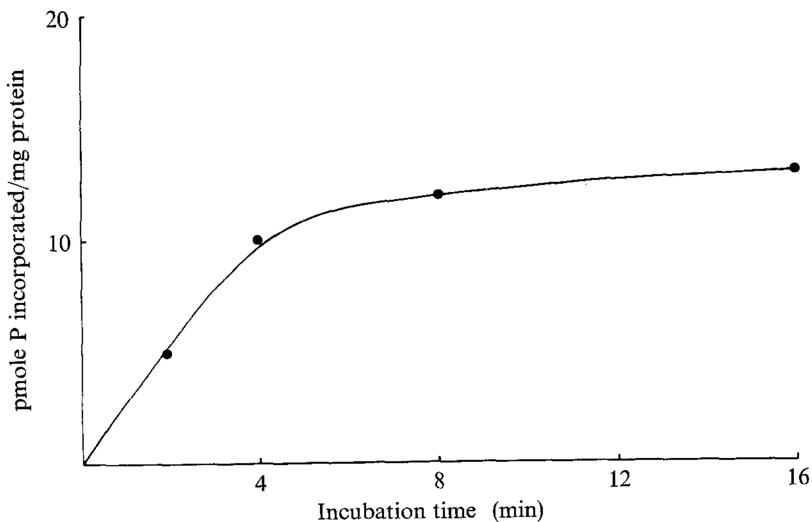


Fig. 2. Cod islet membranes were incubated in assay medium at 15 °C for 2, 4, 8 and 16 min. Total ^{32}P incorporation into protein was then estimated

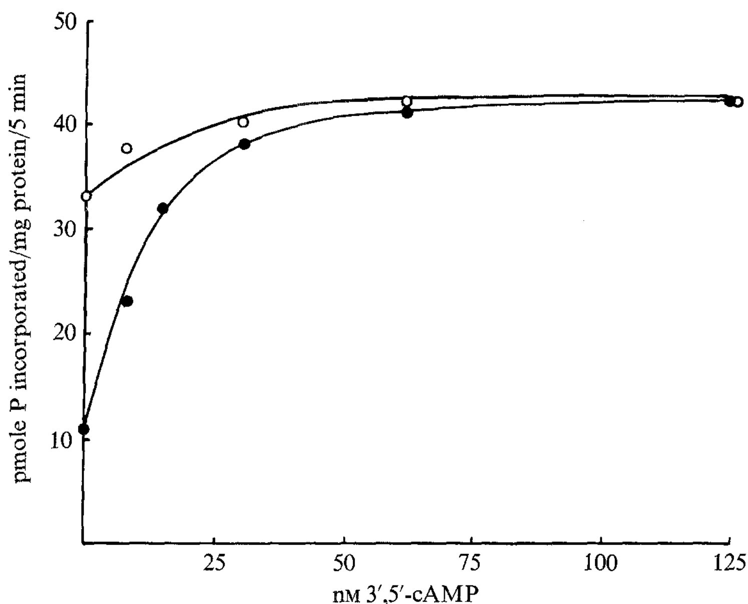


Fig. 3. Cod islet membrane protein phosphokinase was assayed in the presence of 0–125 nM 3′,5′-cAMP and in the absence (●) or presence (○) of 10 mM NaF

cyclase stimulator. Fig. 3 shows that exogenous 3′,5′-cAMP is able to stimulate the enzyme but that this stimulation can be masked by endogenously produced 3′,5′-cAMP when 10 mM fluoride is present. The addition of specific 3′,5′-cAMP antiserum dropped activity to the basal level in the presence or absence of F^-. This shows that the protein kinase is not stimulated by F^-. Nonimmune serum at dilutions of 1:1,000 and 1:10,000 was without effect. Specificity of the antiserum was shown by the fact that 10^{-9} M 3′,5′-cAMP could be displaced only by 10^{-5} M 3′,5′-cGMP or 10^{-3} M 5′-AMP. 5′-ATP did not cross-react with the antibody.

The effect of specific 3′,5′-cAMP antiserum was further examined on the *v vs.s* curves for the enzyme. It may be seen from Fig. 4 that antibody, in the absence of exogenous 3′,5′-cAMP reduced the activity of the enzyme by 75% even at high ATP concentrations. The 3′,5′-cAMP antiserum is thus capable of neutralizing 3′,5′-cAMP produced endogenously and the curve obtained in the presence of antibody represents the basal activity of the enzyme.

Solubilization of Protein Phosphokinase

The cod membrane protein phosphokinase was solubilized by treatment with 0.1% w/v Triton X-100 for 15 min at 15 °C. The 200,000 × *g* pellet showed that considerable activity has been retained on the membrane when

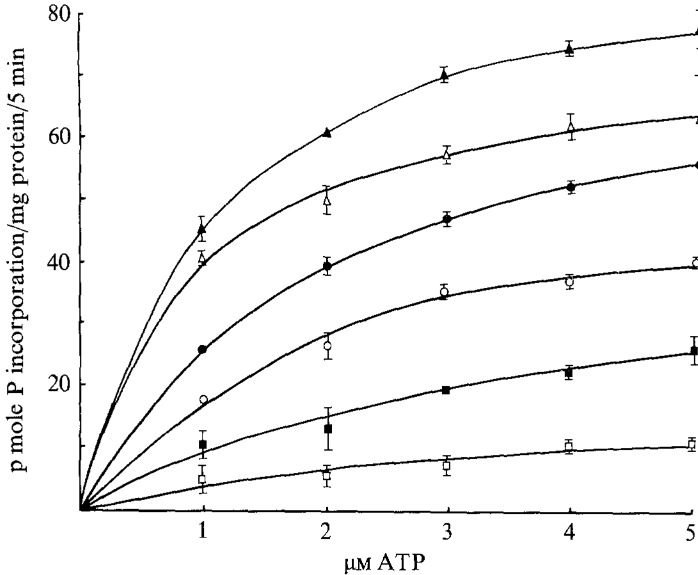
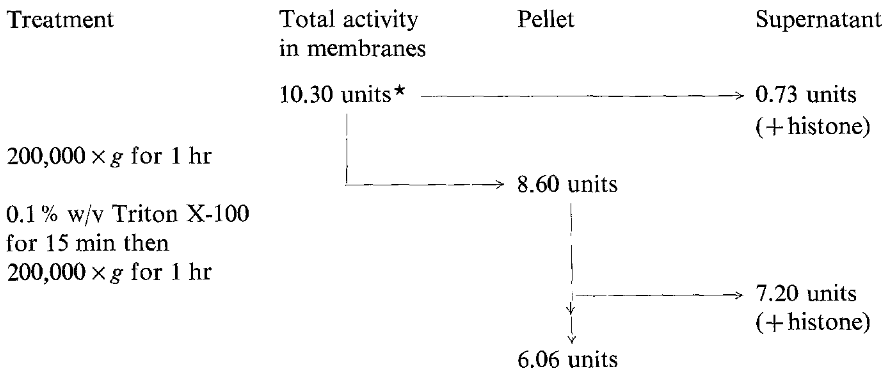


Fig. 4. This figure shows v against s curves for cod islet membrane protein phosphokinase alone (○), with 3′,5′-cAMP antibody (□) and with 125 nM 3′,5′-cAMP (△). This enzyme was also assayed in the presence of 2 mM PEP alone (●), 3′,5′-cAMP antibody with 2 mM PEP (■) and 125 nM 3′,5′-cAMP with 2 mM PEP (▲)

Scheme III

Solubilization of cod islet membranes with 0.1 % w/v Triton X-100. Membranes were solubilized and assayed for protein phosphokinase activity as described in Materials and Methods



assayed with endogenous protein. However, some of the protein phosphokinase had been solubilized as was shown by obtaining measurable enzyme activity in the supernatant on the addition of histone (Scheme III). The

* 1 unit = that amount of enzyme which will transfer 1 pmole P to protein in 5 min at 15 °C.

increase in total enzyme activity is that normally found when a membrane enzyme is solubilized (Lemaire, Pelletier & Labrie, 1971; Rubin, Erlichman & Rosen, 1972).

The K_m for ATP of the tritonated supernatant enzyme was determined by gel filtration on G-200 superfine Sephadex and found to be 166,000. (*c.f.* Montague & Howell (1972), 180,000 for soluble rat islet protein phosphokinase.).

ATPase

The plasma membrane preparation used contained considerable amounts of ATPase activity (140 nmoles ATP hydrolyzed per min/mg protein). Thirty percent of this activity could be inhibited by 5 mM ouabain indicating that 70% of the ATPase activity was not Na^+/K^+ ATPase. The effect of altering ATPase activity on the phosphorylation of the membrane by the protein kinase was investigated. ATPase activity was altered either by the addition of oubain (1 mM) or diphenylhydantoin (0.1 mM) in the presence or absence of 3',5'-cAMP. The effects of these substances are shown in Fig. 5. Ouabain stimulates the protein kinase at all concentrations of ATP below the saturating concentration. This stimulation is seen in the presence

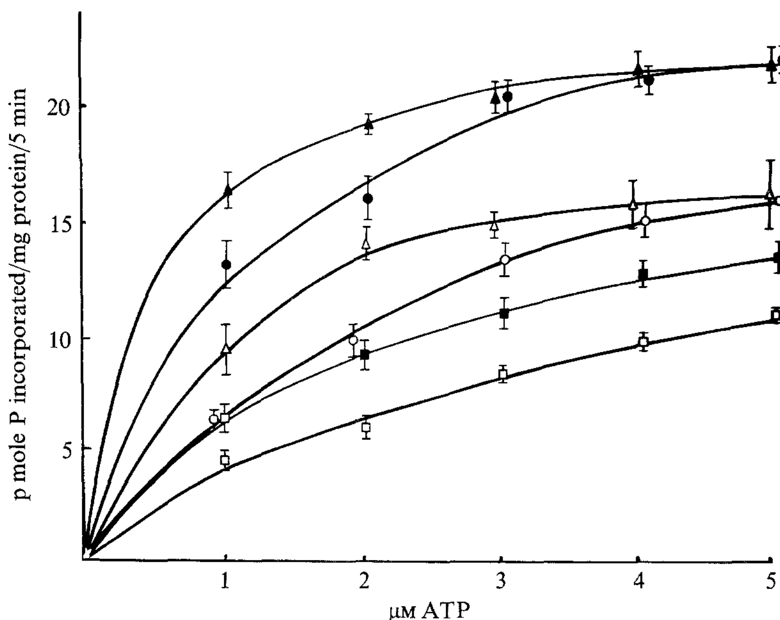


Fig. 5. The effect of ouabain and diphenylhydantoin on the *v* against *s* curves for cod islet membrane protein phosphokinase is shown. (○) control, (●) control with 125 nM 3',5'-cAMP, (△) 1 mM ouabain, (▲) 1 mM ouabain with 125 nM 3',5'-cAMP, (□) 0.1 mM diphenylhydantoin, (■) 0.1 mM diphenylhydantoin with 125 nM 3',5'-cAMP

of saturating amounts of 3′,5′-cAMP. Diphenylhydantoin, a known stimulator of Na^+/K^+ ATPase, causes inhibition of the protein kinase. Ouabain, although causing increased protein kinase activity, did not affect the pattern of ^{32}P incorporation into fractionated membranes.

Effect of Phosphorylated Intermediates of Glucose Metabolism

Cod Islets. The following intermediates of glucose metabolism were tested for activity on the enzyme: glucose-6-phosphate; fructose-6-phosphate; fructose-1-6-diphosphate; glyceraldehyde-3-phosphate; 2-phosphoglycerate; 3-phosphoglycerate; ribose-5-phosphate; pyruvate; phosphoenolpyruvate; 6-phosphogluconate; sorbitol, all at a concentration of 2 mM. Stimulation was only obtained with PEP and G-6-P; results are shown in Fig. 6. This stimulation occurred using both preparations I and II.

Mouse Islets. Both phosphoenolpyruvate and glucose-6-phosphate stimulated the protein phosphokinase. The stimulation obtained was greater

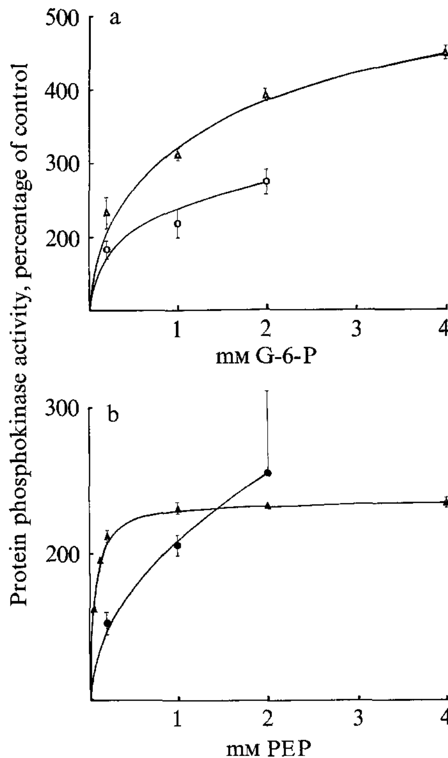


Fig. 6. The effect of the addition of PEP and G-6-P to the normal assay media is shown in this Figure. (*a*) Shows the effect of G-6-P on the activity of the enzyme in preparation I (△) and preparation II (○). (*b*) Shows the effect of PEP in preparation I (▲) and preparation II (●)

Table 2. Effect of PEP and G-6-P on mouse membrane protein phosphokinase activity

Addition	Activity of enzyme (% control)	±SD
2.0 mM PEP	406	29.68
0.2 mM PEP	225	10.49
2.0 mM G-6-P	422	49.62
0.2 mM G-6-P	252	35.63

Mouse membrane protein phosphokinase was assayed in normal assay medium, without the addition of histone; PEP or G-6-P were added at 2.0 and 0.2 mM.

than that obtained for cod islets; PEP and G-6-P at 2 mM gave activities of 406% and 422% of basal, respectively (Table 2).

Nature of the Phosphorylated Membrane Components

In order to investigate the nature of the membrane components which were phosphorylated by the protein phosphokinase, preparations I and II were incubated with [$\gamma^{32}P$] ATP and then solubilized with 0.7% w/v deoxycholate and filtered on G-200 Sephadex.

Fig. 7*a* shows that three components appear to be phosphorylated. A minor peak (Ve/Vo 1.11, peak I) and two other peaks (peak II Ve/Vo 1.61 and peak III Ve/Vo 2.00). The pattern of phosphorylation, when the enzyme was stimulated with phosphoenolpyruvate (using preparation I) and cyclic AMP and glucose-6-phosphate (using preparation II)was identical to the control (Fig. 7*b*, *c* and *d*).

The fractions constituting each peak were pooled, lyophilized and electrophoresed on 15% polyacrylaminde gels. The densitometer and radioactive profiles of these gels are shown in Figs. 8 and 9. Peak I migrates in a position coincident with peak II suggesting that the charge properties of both peaks are identical. Peak III migrates distinct from peak II and is more electropositive than either peak I or peak II.

To determine whether the pattern of phosphorylated substrates obtained on Sephadex filtration could be reproduced by using a different technique, cod islet plasma membranes were first solubilized and then fractionated on a G-200 Sephadex column (*see* method I, solubilization of membranes with 0.7% w/v deoxycholate). Each fraction was then monitored for phosphorus accepting substrate by using purified rat muscle protein phosphokinase. Three peaks of substrate were found. These peaks corresponded in elution position to peaks I, II and III.

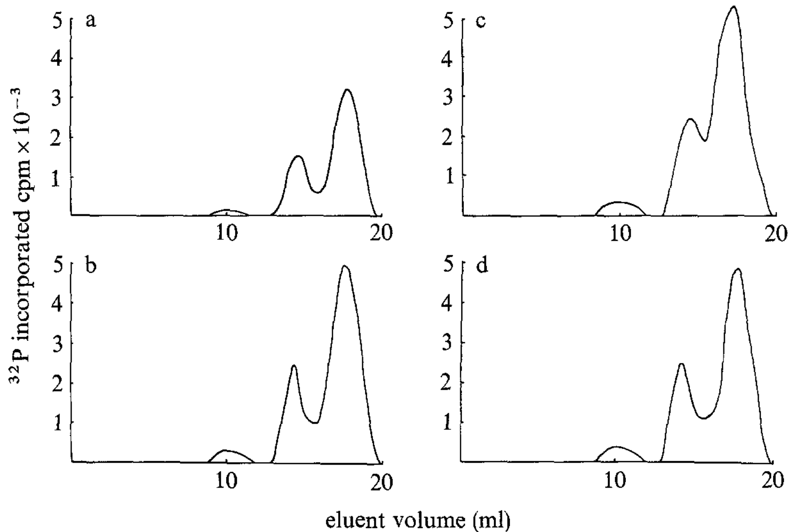


Fig. 7. This Figure shows the ^{32}P incorporation into membranes solubilized with deoxycholate and separated on G-200 Sephadex. (*a*) Shows ^{32}P incorporated from γ^{32}P ATP after incubation in normal assay medium only; membranes from preparation I were used. (*b*) Shows ^{32}P incorporation when 2 mM glucose-6-phosphate was included in the assay medium; membranes from preparation I were used. (*c*) Shows ^{32}P incorporation when 125 nM 3′,5′-cAMP was present; pure preparation II membranes were used. (*d*) Shows ^{32}P incorporation when 2 mM phosphoenolpyruvate was added to the medium; pure preparation II membranes were used

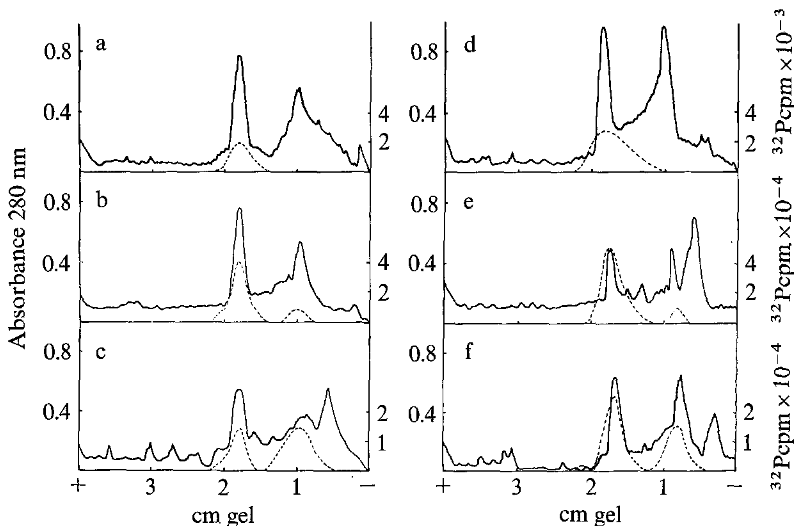


Fig. 8. The fractions constituting peaks I, II and III in Fig. 7 were pooled, lyophilized, dialyzed against running buffer and applied to polyacrylamide gel electrophoresis. (*a*), (*b*) and (*c*) show peaks I, II and III from the unstimulated enzyme. (*d*), (*e*) and (*f*) show peaks I, II and III, respectively, in the presence of phosphoenolpyruvate. Solid lines represent absorbance at 280 nm and broken lines ^{32}P incorporation

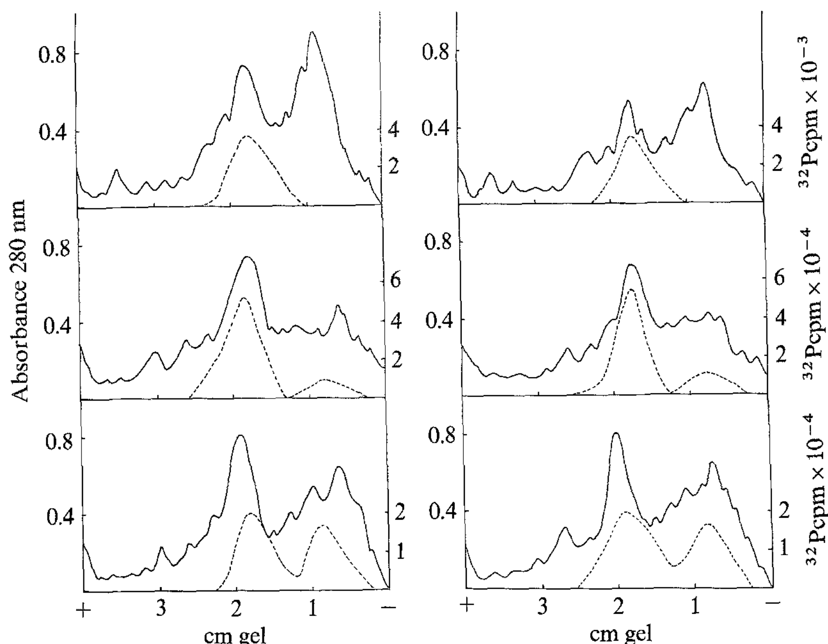


Fig. 9. The fractions constituting peaks I, II and III in Fig. 7*b* and *c* were polled and applied to polyacrylamide gel electrophoresis in the presence of 0.7% w/v deoxycholate. The left-hand Figures show peaks I, II and III in the G-6-P-stimulated enzyme. The right-hand Figures show peaks I, II and III in the presence of 3′,5′-cAMP. Solid lines represent absorbance at 280 nM and broken lines ^{32}P incorporation

Discussion

The plasma membranes utilized in this study were identified by enzyme marker activities as well as by electron-microscopy. Purification of the plasma membrane by 0.7% w/v deoxycholate followed by 48-hr dialysis did not alter the properties of the enzyme which were identical to those found in membranes purified by density gradient centrifugation. The plasma membranes contained in addition to protein kinase activity, adenylate cyclase, phosphodiesterase and ATPase activities. The presence in the membrane of both adenylate cyclase and phosphodiesterase suggests that concentrations of 3′,5′-cAMP at the membrane can rise without necessarily affecting intracellular levels. Approximately 30% of the ATPase could be inhibited by ouabain. The small amounts of mouse islet tissue available (1 islet $\simeq$ 0.6 μg protein) caused the bulk of this work to be carried out on cod islet tissue (1 cod islet $\simeq$ 100 mg).

Cod insulin is stored in β-granules and synthesized via proinsulin (Grant & Reid, 1968). The β-granules of another species of fish *(Lophius*

americanus) are similar in structure and behavior to rat granules (Lindall, Bauer, Dixit & Lazarow, 1963; Howell, Fink & Lacy, 1969; Sorenson, Lindall & Lazarow, 1969). It may therefore be expected that the mechanisms associated with extrusion of β-granules are similar. In addition, cod and mouse islet membrane protein phosphokinase have exhibited similar properties when tested under similar experimental conditions. The pH optima of both enzymes was the same. Neither was stimulated by glucose. Both are stimulated by endogenous and exogenous 3′,5′-cAMP as was demonstrated in the experiments utilizing fluoride and specific 3′,5′-cAMP antibody. Because cod islets are not rich in β-cells (45%) the effects of G-6-P and PEP were tested on mouse membranes.

It was found that both mouse and cod enzymes were stimulated by glucose-6-phosphate and phosphoenolpyruvate. This stimulation was demonstrated in the absence of 3′,5′-cAMP (i.e. in the presence of specific 3′,5′-cAMP antibody) or in the presence of saturating levels of 3′,5′-cAMP. Glucose is a potent stimulator of insulin release and it may be that glucose is coupled to events at the membrane by such intermediates. These results suggest that the cod provides a convenient model in which to try and understand the mechanisms of insulin release. The cod islet protein kinase is not inhibited by theophylline (*see* Weller & Rodnight, 1973). Fluoride ions were also without effect on the enzyme.

Ouabain potentiates the effect of glucose on both phases of insulin release (Gutman & Lazarus, *unpublished observation*). It is also a potent inhibitor of (Na^+/K^+) ATPase. This effect could be demonstrated in the present study. Ouabain caused stimulation of the protein phosphokinase activity either in the absence or presence of saturating levels of 3′,5′-cAMP. The ouabain effect is thus not mediated via the cyclase system. Ouabain only stimulated the protein phosphokinase at concentrations of ATP that were below saturation. Thus, ouabain may be providing the kinase with more substrate by inhibiting (Na^+/K^+)-ATPase activity.

The membrane-bound protein phosphokinase was unable to phosphorylate exogenous substrate, e.g. histone. When the enzyme was solubilized, histone was phosphorylated. Mild detergent treatment was able only to solubilize the protein phosphokinase activity and did not solubilize what appeared to be specific substrate or substrates for the enzyme. The phosphorylated components in the membrane were partially identified by Sephadex filtration and polyacrylamide gel electrophoresis. The polyacrylamide gel patterns show that peaks I and II have similar migratory properties and thus may be composed on similar subunits. Peak III has different

migratory properties. The significance of both these species is at present unknown.

We are unable to definitively assert that phosphoenolpyruvate, glucose-6-phosphate and cyclic AMP all exert their activity via the same protein kinase. However, as can be seen from Fig. 7 all these substances produce the same pattern of ^{32}P incorporation into membrane protein. Secondly, polyacrylamide electrophoresis of the radioactive peaks show identical patterns.

References

Bowen, V., Lazarus, N. R. 1973. Glucose-mediated insulin release: 3′,5′-cAMP phosphodiesterase. *Diabetes* **22:**738

Cooperstein, S. J., Lazarow, A. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189:**665

Davis, B., Lazarus, N. R. 1972. Insulin release from mouse islets: Effect of glucose and hormones on adenylate cyclase. *Biochem. J.* **129:**373

Fiske, C. H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:**375

Freeman, J. A., Spurlock, B. O. 1962. A new epoxy-embedment for electron-microscopy. *J. Cell Biol.* **13:**437

Grant, P. T., Reid, K. B. M. 1968. Biosynthesis of an insulin precursor by islet tissue of cod (*Gadus callarius*). *Biochem. J.* **110:**281

Howell, S. L., Fink, C. J., Lacy, P. E. 1969. Isolation and properties of secretory granules from rat islets of Langerhans. *J. Cell Biol.* **41:**154

Howell, S. L., Taylor, K. W. 1968. Potassium ions and the secretion of insulin by islets of Langerhans incubated *in vitro*. *Biochem. J.* **108:**17

Johnson, E. M., Maeno, H., Greengard, P. 1971. Phosphorylation of endogenous protein of rat brain by cyclic adenosine 3′,5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **246:**7731

Kiehn, E. D., Holland, J. J. 1970. Membrane and nonmembrane proteins of mammalian cells. Synthesis, turnover and size distribution. *Biochemistry* **9:**1716

Kuo, J. F., Krueger, B. K., Sanes, J. R., Greengard, P. 1970. Cyclic nucleotide-dependent protein kinases. V. Preparation and properties of adenosine 3′,5′-monophosphate-dependent protein kinase from various bovine tissues. *Biochim. Biophys. Acta* **212:**79

Lemaire, S., Pelletier, G., Labrie, F. 1971. Adenosine 3′,5′-monophosphate-dependent protein kinase from bovine anterior pituitary gland. II. Subcellular distribution. *J. Biol. Chem.* **246:**7303

Lindall, A. W., Bauer, G. E., Dixit, P. K., Lazarow, A. 1963. Isolation of an insulin secretion granule fraction. *J. Cell Biol.* **19:**317

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265

Montague, W., Howell, S. L. 1972. The mode of action of adenosine 3′:5′-cyclic monophosphate in mammalian islets of Langerhans. Preparation and properties of islet-cell protein phosphokinase. *Biochem. J.* **129:**551

Reynolds, E. S. 1963. The use of lead acetate at high pH on electron-opaque stain in electron microscopy. *J. Cell Biol.* **17:**208

Rubin, C. S., Erlichman, J., Rosen, O. M. 1972. Cyclic adenosine 3′,5′ monophosphate-dependent protein kinase of human erythrocyte membrane. *J. Biol. Chem.* **247**:6135

Sorenson, R. L., Lindall, A. W., Lazarow, A. 1969. Studies on the isolated goosefish insulin secretion granule. *Diabetes* **18**:129

Weller, M., Rodnight, R. 1973. State of phosphorylation *in vivo* of membrane-bound phosphoproteins in rat brain. *Biochem. J.* **133**:387