Biochem. J. (1978) **176**, 855–863
Printed in Great Britain

Control of Amylase Biosynthesis and Release in the Parotid Gland of the Rat

By MARGARET A. McPHERSON* and C. NICHOLAS HALES†
Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K.

(*Received* 24 *July* 1978)

1. Amylase biosynthesis and release in the rat parotid were studied under various conditions. Incorporation of [^{3}H]leucine into amylase, extracted from the tissue by immunoadsorbent, was measured and found to be time-dependent and totally inhibited by the protein synthesis inhibitor puromycin. 2. Adrenaline, at a concentration (10μM) that gave maximum stimulation of release, inhibited [^{3}H]leucine incorporation into both total protein and amylase. This effect was reversed by phentolamine. 3. Adrenaline (1μM) and isoproterenol (10μM) stimulated biosynthesis of total protein and amylase. These effects were blocked by propranolol, as were the effects on release. Dibutyryl cyclic AMP (2mM) mimicked the effects of isoproterenol and adrenaline (1μM) on both amylase biosynthesis and release. All the above stimulatory effects on amylase biosynthesis were only observed if the tissue was pretreated with effector before pulse-labelling with [^{3}H]leucine. 4. Insulin (625μunits/ml initial concentration, 150μunits/ml final concentration) stimulated incorporation of [^{3}H]leucine into total protein and amylase when added to the tissue at the same time as the leucine. 5. Carbamoylcholine (10μM) decreased [^{3}H]leucine incorporation into total protein and amylase when both were added to the tissue simultaneously, but this effect was prevented by removal of effector and washing the tissue before addition of [^{3}H]leucine. 6. Stimulation of β-adrenergic receptors increased both amylase release and biosynthesis, but stimulation of α-receptors can inhibit biosynthesis without inhibiting release. Cholinergic agents can also inhibit amylase biosynthesis, but stimulate release. Insulin at approximately physiological concentration can increase incorporation of leucine into amylase without stimulating release. The system described therefore provides an excellent model for the further investigation of the mechanisms of these diverse effects.

In a number of polypeptide secretory systems some, but not all, of the stimulators of secretion also increase the biosynthesis of the secreted polypeptide. Glucose stimulates insulin secretion and biosynthesis (Taylor *et al.*, 1964), whereas the drug tolbutamide stimulates insulin secretion, but not its biosynthesis (Taylor & Parry, 1967). Adrenaline via an α-receptor inhibits insulin secretion, whereas adrenergic activation of a β-cell catecholamine β-receptor potentiates glucose-stimulated insulin secretion. Adrenaline alone or isoproterenol in the absence or presence of a β-blocker has no effect on glucose-stimulated insulin biosynthesis (Lin & Haist, 1973, 1975).

In the exocrine pancreas, stimulators of amylase release increase amylase biosynthesis (Rosenfeld *et al.*, 1976). The release of amylase from the rat parotid gland is regulated by both α- and β-adrenergic and cholinergic agents (Bdolah *et al.*, 1964; Schneyer & Hall, 1966; Batzri *et al.*, 1971; Schramm & Selinger, 1975). The presence of these different control mechanisms together with the homogeneity of the tissue, great preponderance of amylase in the secretion and availability of reasonable quantities of tissue make this gland a good system in which to study the coupling of polypeptide secretion to synthesis. Grand & Gross (1969) reported that adrenaline and dibutyryl cyclic AMP increase the biosynthesis of total protein and amylase in the rat parotid by using glycogen precipitation to partially purify amylase, but without demonstrating homogeneity of the extracted amylase. In view of the frequent existence of biosynthetic precursors to secreted polypeptides (Campbell & Blobel, 1976), careful characterization of newly synthesized polypeptide is now essential.

The present paper describes an immunological method for the isolation and characterization of newly synthesized amylase from the rat parotid gland. The relationship between total protein biosynthesis and amylase biosynthesis and release was studied by using α- and β-adrenergic stimulation,

* Present address: Hormone Research Laboratory, University of California, San Francisco, CA 94143, U.S.A.
† Present address: Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

dibutyryl cyclic AMP, carbamoylcholine and insulin.

Experimental

Materials

Adrenaline, which was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., was made up immediately before use and dissolved in HCl (0.1M); bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; carbamoylcholine chloride, crystalline bovine insulin (24units/mg) and phenylmethanesulphonyl fluoride were from Sigma, St. Louis, MO, U.S.A.; DEAE-Sephadex and Sephadex G-100 were from Pharmacia, London W5 5SS, U.K.; 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were from Nuclear Enterprises, Sighthill, Edinburgh 11, Scotland, U.K.; [4,5-^{3}H]leucine (sp. radioactivity 53Ci/mmol was from The Radiochemical Centre, Amersham, Bucks., U.K.; microgranular cellulose powder (CC41) from Whatman Biochemicals, Maidstone, Kent, U.K.; phentolamine mesylate [2-*N*-(3-hydroxyphenyl)-*p*-toluidinomethyl-2-imidazoline mesylate; Rogitine] was from Ciba Laboratories, Horsham, Sussex, U.K.; propranolol was from ICI Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire, U.K.

All other reagents were purchased from BDH Chemicals, Poole, Dorset, U.K., and were of the highest purity available.

Methods

Assay of amylase. Amylase activity was assayed by the method of Bernfeld (1955). Incubations were for 15min. One unit of amylase activity is defined as the amount of enzyme required to liberate 1.0μmol of maltose equivalent/min at 25°C.

Purification of amylase. Amylase was purified by the method described by Ball (1974) from the parotid glands of 12 (250–300g) male Wistar rats that had been starved overnight. The final freeze-dried product had a specific activity of 1260units/mg of protein. At 30°C it liberated 2700mg of maltose in 3min/mg of protein, which compares with values ranging from 2500 to 3000 reported for purified rat parotid-gland amylases by other workers (Ball, 1974; Messer & Dean, 1975). When electrophoresed on 7.5% (w/v) polyacrylamide gels in Tris/glycine buffer, pH8.3 (Davis, 1964), the major component observed after staining with Amido Black or Coomassie Brilliant Blue had an R_F of 0.39–0.41, which is the same as that reported by Ball (1974). On application of at least 200μg of protein to the gels, four trace minor components were also visible, the gel pattern being similar to that obtained by Messer & Dean (1975) who ran 7% polyacrylamide gels in a similar buffer system. Duplicate gels could be stained intact for amylase activity by immersion in starch solution (1% in amylase assay buffer) for 15min at 25°C followed by immersion in alkaline 1% dinitrosalicylic acid solution and boiling for 5min (see Bernfeld, 1955). All of the protein bands observed after staining with Coomassie Brilliant Blue also stained for amylase activity, in agreement with Messer & Dean (1975).

Raising of antisera. Amylase from rat parotid gland, purified as described above, was used as antigen. Antisera were raised in New Zealand White rabbits by repeated multiple-site intradermal injections of 20–50μg of protein in 1.0ml of a 1:1 mixture of Freund's adjuvant and potassium phosphate buffer (50mM, pH7.4). Injections were given at four or five weekly intervals, Freund's complete adjuvant being used only for the first injections. Rabbits were bled from an ear vein 10 days after the third and subsequent injections.

Preparation of immunoadsorbents. A crude immunoglobulin fraction was prepared from antisera or control rabbit sera by precipitation with 18% Na_2SO_3, washing the precipitate once and redissolving in borate buffer (0.2M, pH8.2). The immunoglobulin fraction was dialysed overnight at 4°C against borate buffer (0.2M, pH8.2) with three changes of dialysis medium. The protein content of the non-diffusible material was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as standard. Crude immunoglobulin fraction 10–15mg was obtained from 1ml of antiserum.

Immunoadsorbents were prepared by coupling the immunoglobulin fraction to a diazonium derivative of powdered cellulose (Whatman CC 41) by the method of Gurvich *et al.* (1961) as described by Miles & Hales (1968). The diazocellulose suspension (40mg/ml in 0.2M-borate buffer, pH8.2) was added to crude immunoglobulin fraction to give a 3:1 (w/w) ratio of cellulose/protein. After rotating the mixture for 48h at 4°C in the dark, the antibody-coupled cellulose was washed with 3×50ml of potassium phosphate buffer (100mM, pH7.5, containing 100mM-NaCl), resuspended in 20–30ml of the same buffer containing 0.01% sodium azide and stored in the dark at 4°C. With the above ratio of diazocellulose/antibody, 50–60% of the total protein in the crude immunoglobulin fraction was coupled (i.e. 170–180μg of protein was coupled per mg of cellulose base). Before quantities of immunoadsorbent were used for the extraction of amylase from parotid tissue, the immunoadsorbent was always washed with 3×10ml of homogenizing buffer, with centrifugation at 300g for 2min after each wash.

Incubation medium. Leucine-free Eagle's basal medium (Wellcome Research Laboratories, Becken-

ham, Kent, U.K.), containing 5.5mM-glucose and gassed with O_2/CO_2 (19:1 v/v), pH7.4, was used for all tissue incubations. Any additions to this medium are stated in the text, Tables and Figures.

Tissue incubation procedure. Parotid glands were dissected from male Wistar rats (160–200g) that had been starved overnight, and killed by stunning and decapitation. The glands were placed in incubation medium at 37°C and cut with scissors into small pieces weighing 15–25mg. The parotid pieces were preincubated (one per vial containing 2ml of incubation medium) for 30min at 37°C. After removal of this medium, the tissue was incubated either for 60min in 2ml of incubation medium containing [4,5-^{3}H]leucine (20μCi or 50μCi per vial), or for 60min in 2ml of non-radioactive medium, then for a further 20min in 2ml of fresh medium containing [4,5-^{3}H]leucine (20μCi per vial) (pulse-labelling method). Any additions to the medium and the incubation procedure adopted are stated in the text, Tables and Figures.

At the end of the incubation, the medium was removed with a Pasteur pipette and the tissue washed with 3×2ml of ice-cold incubation medium containing 0.4mM-leucine, weighed and homogenized in 0.75ml of homogenizing buffer (50mM-Tris/HCl containing 1mM-phenylmethanesulphonyl fluoride and 5% propan-2-ol, pH7.5). Samples were taken for treatment with trichloroacetic acid and determination of protein content. The remaining homogenates were centrifuged at 10000g for 2min in an Eppendorf bench centrifuge, amylase being extracted from the supernatant. Incubation media and centrifuged homogenates were diluted and assayed for amylase activity.

Extraction of protein and amylase. Protein was precipitated from 100μl of tissue homogenate by the addition of an equal volume of ice-cold 20% (w/v) trichloroacetic acid. The precipitates were washed with 3×500μl of ice-cold 10% (w/v) trichloroacetic acid and dissolved in 200μl of 1M-NaOH (60°C for 30min). After cooling, the radioactivity of a sample was counted in toluene/Triton X-100 scintillation fluid (2 litres of toluene; 1 litre of Triton X-100; 8g of PPO; 0.2g of POPOP) in a Packard liquid-scintillation spectrometer.

Amylase was extracted from 50μl (of a 1:10 dilution) of centrifuged parotid homogenate by incubation with 200μl of immunoadsorbent suspension in homogenizing buffer (containing 680–700μg of crude immunoglobulin fraction coupled to 4mg of cellulose) for 2h at 20°C. The immunoadsorbents were then centrifuged (10000g for 2min) and samples of the supernatant were taken for amylase assay and for counting of radioactivity. The immunoadsorbents were washed with 3×500μl of homogenizing buffer, dissolved in 250μl of PCS tissue solubilizer, phase scintillator (Amersham/Searle, Amersham, Bucks., U.K.) and counted for radioactivity in the toluene/Triton X-100 scintillation fluid.

Elution of radioactive material bound to immunoadsorbent for polyacrylamide-gel electrophoresis. Homogenizing buffer (100μl) containing 1mg of Na_2CO_3, 25μl of 10% sodium dodecyl sulphate and 14μl of β-mercaptoethanol was added and the mixture heated for 5–10min at 100°C. Spacergel buffer (90μl) (54mM-Tris/H_2SO_4, pH6.1), 45μl of 40% sucrose and 70μl of water were then added. The tubes were centrifuged (300g for 2min) and 30μl of the supernatant was applied to 7.0% polyacrylamide gels and run in a sodium dodecyl sulphate buffer system (running pH9.5) as described by Neville (1971). The gels were frozen and cut into 1.5mm segments. Each segment was dissolved by heating at 60°C for 18h in 200μl of 30% H_2O_2 and counted for radioactivity in the toluene/Triton X-100 scintillation fluid.

In most experiments radioactive amylase was extracted from the tissue by the above immunoprecipitation method. However, in some experiments the radioactivity incorporated into amylase was measured by running a sample of centrifuged parotid homogenate on 7.5% polyacrylamide gels (Davis, 1964). The amylase band identified by its R_F and amylase activity (see above) was cut out, and dissolved and counted in the same way as were the sodium dodecyl sulphate/polyacryamide-gel slices. Where this method has been used to determine incorporation of radioactivity into amylase, this is stated in the text.

Determination of radioactivity. The counting efficiency for ^{3}H was 20–30% and in all cases quench corrections were made by an internal channels-ratio method.

Results

Capacity and specificity of immunoadsorbents

Fig. 1 shows the result of incubating various amounts of immunoadsorbent with either 1 unit of pure amylase preparation (approx. 0.8μg), or with a sample (5μl) of centrifuged parotid homogenate. Amylase activity was completely inhibited both in the pure preparation and in the parotid homogenate by 200μl of immunoadsorbent suspension [containing approx. 700μg of crude immunoglobulin (prepared from approx. 50μl of antiserum, as described under 'Methods') coupled to 4mg of cellulose]. Control rabbit immunoglobulin-coupled cellulose did not inhibit either pure amylase activity or amylase activity in parotid homogenates.

To characterize the material extracted from parotid tissue by the immunoadsorbent, tissue was incubated with [^{3}H]leucine for 60min, homogenized, centrifuged and the homogenate either run directly on sodium

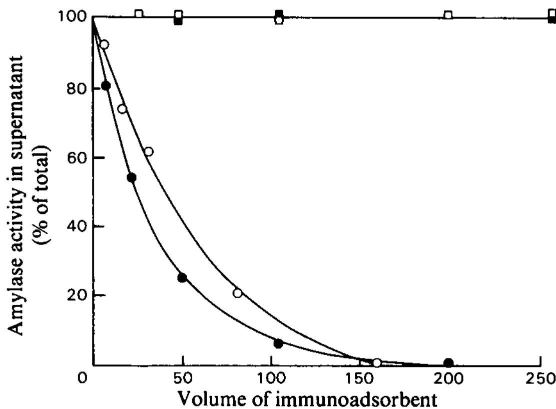

Fig. 1. *Inhibition of amylase activity by antibody-coupled immunoadsorbent*

Various amounts of anti-amylase immunoadsorbent were incubated for 2h at 20°C with either 5μl of centrifuged parotid homogenate (●) or 1 unit of pure amylase (○). After centrifuging at 300g for 2min, the amylase activity of the supernatant was measured. Incubation of parotid homogenate (■) or pure amylase (□) with immunoadsorbent to which non-immune immunoglobulin has been coupled is also shown. Immunoadsorbent suspension (200μl) contained 0.7mg of crude immunoglobulin coupled to 4mg of cellulose.

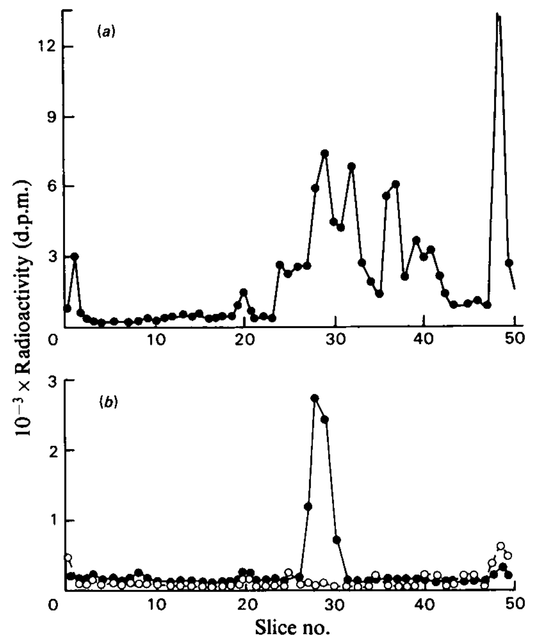

Fig. 2. ^3H *radioactivity profiles from sodium dodecyl sulphate/polyacrylamide gels*

Centrifuged homogenates (100μl) of parotid tissue that had been incubated with [^{3}H]leucine were either treated with sodium dodecyl sulphate and run on polyacrylamide gels as described under 'Methods' (a) or were incubated with 1 ml of anti-amylase immunoadsorbent, which removed 30% of the total amylase activity from the homogenate. The material extracted by the immunoadsorbent was eluted with sodium dodecyl sulphate as described under 'Methods' and run on polyacrylamide gels (●; b). The gel profile of material extracted by incubation of parotid homogenate with non-immune immunoglobulin-coupled cellulose (○; b) is also shown.

dodecyl sulphate/polyacrylamide gels (Fig. 2a) or treated with immunoadsorbent. The bound material was subsequently eluted with sodium dodecyl sulphate (see under 'Methods'). Fig. 2(b) shows the radioactivity profiles obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of material eluted from immunoadsorbent (anti-amylase) or from control immunoglobulin coupled cellulose (non-immune). With anti-amylase immunoadsorbent a major peak of radioactivity was eluted that had an R_F value identical with that of pure amylase, run under similar conditions. A corresponding peak of radioactivity was also present in the profile of untreated parotid homogenates. A small amount of radioactivity was eluted from control immunoglobulin-coupled cellulose, most of this running with the ion front on sodium dodecyl sulphate/polyacrylamide gels.

Time course of [^3H]*leucine incorporation*

A linear rate of incorporation of [^{3}H]leucine into both trichloroacetic acid-precipitable proteins and amylase was observed over a 60min incubation period (Fig. 3) after a slight time lag of 5–10min, which was observed in both cases. This slower initial rate may reflect the time taken for proteins large enough to be precipitated by trichloroacetic acid to be synthesized. The incorporation of [^{3}H]leucine into amylase was approx. 30% of that into total trichloroacetic acid-precipitable protein. Incorporation of radioactivity into both total protein and amylase was rapidly abolished in the presence of puromycin, an inhibitor of protein synthesis.

Effects of stimulators of amylase release on [^3H]*leucine incorporation*

Adrenaline was able to increase amylase release from parotid tissue by 7–8-fold, but markedly decreased [^{3}H]leucine incorporation into both total protein and amylase (Table 1). The concentration of adrenaline giving maximum stimulation of amylase release was 10μM, but that giving maximum inhibition of [^{3}H]leucine incorporation into amylase was 100μM.

Other potent stimulators of amylase release such as isoproterenol (10μM) and dibutyryl cyclic AMP (2mM) had no effect on [^{3}H]leucine incorporation into total protein or amylase, but carbamoylcholine (10μM), like adrenaline decreased the incorporation rate (Table 2).

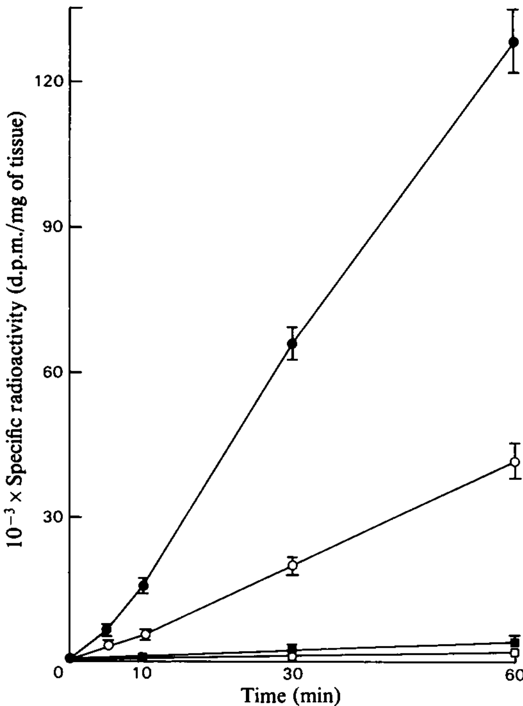

Fig. 3. *Time course of [^{3}H]leucine incorporation into total protein and amylase*

Parotid tissue (20mg) was incubated with 50μCi of [^{3}H]leucine for various times in the presence (□, ■) or absence (●, ○) of puromycin (200μg/ml). The radioactivity incorporated into total protein (●, ■) (trichloroacetic acid precipitation method) and amylase (○, □) (immunoprecipitation method) is shown. Results are means±S.E.M. for eight observations.

Effects of insulin

Insulin in the concentration range 100–2500 μunits/ml stimulated the incorporation of [^{3}H]-leucine into both total protein and amylase in a dose-dependent manner (Table 3). It had no effect on

Table 1. *Effect of adrenaline on amylase release and [^{3}H]leucine incorporation into trichloroacetic acid-precipitable protein and amylase*

Parotid tissue (20mg) was incubated with 50μCi of [^{3}H]leucine for 60min in the presence of various concentrations of adrenaline. The incubation medium also contained unlabelled (0.4mM) leucine. The amount of amylase released into the incubation medium is shown and also the radioactivity incorporated into total protein and into amylase. In these experiments, incorporation of radioactivity into amylase was measured by polyacrylamide-gel electrophoresis of centrifuged parotid homogenate, cutting out the amylase band and counting the radioactivity (see under 'Methods'). Results (expressed as % of control) are means±S.E.M. for four observations. Control incorporation into trichloroacetic acid precipitates was 17150–34500d.p.m./mg of tissue and into amylase was 6400–14800d.p.m./mg of tissue. Control rates of amylase release varied from 0.30 to 0.42 unit/mg of tissue.

[Adrenaline] (μM)	Amylase release (% of control)	Radioactivity incorporated (% of control)	
		Total Protein	Amylase
0	100±9	100.0±7.9	100.0±7.1
10	510±123	58.7±6.1	38.2±11.0
50	565±71	13.1±1.8	5.8±1.8
100	476±42	6.1±0.7	3.4±0.5
1000	434±27	6.5±0.7	1.9±0.3

Table 2. *Effects of isoproterenol, dibutyryl cyclic AMP and carbamoylcholine on amylase release and [^{3}H]leucine incorporation into total protein and amylase*

Results are means±S.E.M. for parotid pieces preincubated for 20min in control Eagle's medium and then incubated for 60min in the presence of 50μCi (in experiments where isoproterenol and dibutyryl cyclic AMP were used) or 20μCi (in experiments with carbamoylcholine) of [^{3}H]leucine, under the conditions shown. Values in parentheses denote the numbers of observations. ***$P<0.001$ for difference from control by Student's t test.

	Amylase release (units/mg of tissue)	Radioactivity (d.p.m./mg of tissue)		
		Trichloroacetic acid precipitate	Amylase	
Control	0.51±0.09	109247±10127	36190±4743	(10)
Isoproterenol (10μM)	2.81±0.30***	116510±8783	38990±3313	(10)
Control	0.23±0.03	144827±9467	54790±4367	(7)
Dibutyryl cyclic AMP (2mM)	0.87±0.05***	130533±9390	53890±2643	(7)
Control	0.16±0.04	74630±3770	24220±1623	(6)
Carbamoylcholine (10μM)	1.10±0.24***	17013±2300***	6133±777***	(6)

amylase release at any of the concentrations used, except in one group of experiments where a significant stimulation (146±14%, $n=14$, $P<0.01$) at a concentration of 625 μunits of insulin/ml was observed. At the end of the incubation samples were withdrawn for radioimmunoassay (Hales & Randle, 1963) of their insulin content. It was found that the final insulin content was only 25% of that added initially. Thus the average concentration to which the tissue was exposed was considerably lower than that indicated, suggesting that these effects could well be of physiological significance.

Pulse-labelling experiments

In this series of experiments, the tissue was incubated with various agents for 60min, before the addition of [^{3}H]leucine (see under 'Methods').

With this procedure, isoproterenol (10μM) increased the incorporation of [^{3}H]leucine into both total protein (by 156%) and amylase (by 190%). These effects were decreased by propranolol, a β-adrenergic blocker, as were the stimulatory effects of isoproterenol on amylase release (Table 4). When phentolamine, an α-adrenergic blocker, was present together with isoproterenol, an enhancement of the stimulation with isoproterenol alone was observed, but this was not significant.

Dibutyryl cyclic AMP had similar effects to isoproterenol. Thus it increased incorporation of [^{3}H]leucine into amylase to a greater extent than into trichloroacetic acid-precipitable protein (Table 5). Stimulation of amylase release and of [^{3}H]leucine incorporation were observed after pretreatment of tissue with dibutyryl cyclic AMP for 60min, whether or not this effector was included in the final 20min [^{3}H]leucine pulse incubation period (Table 5).

The inhibitory effect of adrenaline on [^{3}H]leucine incorporation (Table 1) was again observed during pulse-labelling experiments where adrenaline was included in both 60 and 20min ([^{3}H]leucine pulse) incubation periods. This effect could be reversed by including phentolamine in both incubation periods (Table 6). In some experiments, the inclusion of phentolamine with adrenaline resulted in a stimulation above control, but the overall effect was not

Table 3. *Effects of insulin on [^{3}H]leucine incorporation into total protein and amylase*

Parotid tissue was incubated for 60min with 50μCi of [^{3}H]leucine in the presence or in the absence of insulin. The concentration of insulin added at the beginning of the experiment is shown. However, the amount of insulin in the incubation medium at the end of the incubation, measured by radioimmunoassay, was approx. 25% of the initial concentration. Thus on average over 1h of incubation the tissue was exposed to much lower insulin concentrations than those shown in the Table. Results, expressed as % of control, are means±S.E.M. for six observations. Control incorporation of radioactivity into total protein varied from 92800 to 19300d.p.m./mg of tissue and into amylase from 31200 to 62100d.p.m./mg of tissue. The significance of differences was obtained by using Student's t test: †$P<0.05$; *$P<0.02$; **$P<0.005$; ***$P<0.001$ for difference from control.

Insulin concn. (μunits/ml)	Incorporation of radioactivity (expressed as % of control)	
	Total protein	Amylase
0	100±4	100±5 (6)
10	112±8	124±10 (6)
100	115±3	114±6 (6)
625	153±6***	138±15 (6)†
1000	150±16*	155±16 (6)†
2500	153±13**	128±6 (6)*

Table 4. *Pulse-labelling experiments: effects of pretreatment of parotid tissue withi soproterenol and propranolol before addition of [^{3}H]leucine*

Parotid tissue was incubated for 60min under the conditions in Table 3. The tissue was then pulsed with 20μCi of [^{3}H]leucine for 20min under the same incubation conditions. The amount of amylase released during both incubation periods (a linear rate was observed over 80min) is expressed as % of control. Control amylase release varied from 0.30 to 1.11units/60min per mg of tissue. Control incorporation of [^{3}H]leucine into trichloroacetic acid precipitates was 10106–20423d.p.m/mg of tissue and into amylase was 3145–7500d.p.m./mg of tissue. Results are means±S.E.M. Values in parentheses denote the numbers of observations. The significance of differences was obtained by Student's t test: ***$P<0.001$ for difference from control; **$P<0.005$ for difference from tissue incubated in the presence of propanolol alone.

Additions (μM)	Amylase released (% of control)	Radioactivity incorporated (% of control)	
		Total protein	Amylase
None	100±9 (9)	100±2 (9)	100±3 (9)
Isoproterenol (10)	410±40*** (9)	156±5*** (6)	186±9*** (6)
Propranolol (30)	112±6 (5)	106±7 (5)	108±5 (5)
Isoproterenol (10)+propranolol (30)	142±20 (5)	119±6 (5)	129±7** (5)

Table 5. *Pulse-labelling experiments: pretreatment of parotid tissue with dibutyryl cyclic AMP for 60min, before addition of [³H]leucine*

Results are means ± S.E.M. (numbers of observations in parentheses) for parotid pieces preincubated for 30min in control Eagles medium and then incubated in the presence or in the absence of dibutyryl cyclic AMP (2mM) for 60min. The tissue was then pulsed for 20min with 20μCi of [³H]leucine, in the presence or in the absence of dibutyryl cyclic AMP. Results are expressed as % of controls. Control amylase release varied from 0.15 to 0.43 unit/60min per mg of tissue, and control incorporation into trichloroacetic acid precipitates from 6511 to 10400d.p.m./mg of tissue and into amylase from 2396 to 3921 d.p.m./mg of tissue. *$P<0.02$; **$P<0.005$ for difference from control by Student's t test. Difference between incorporation into trichloroacetic acid precipitates and amylase was significant: $P<0.01$ (dibutyryl cyclic AMP present in both incubation periods), $P<0.05$ (dibutyryl cyclic AMP absent from second incubation period). Difference between incorporation of radioactivity into trichloroacetic acid or amylase, in the presence or in the absence of dibutyryl cyclic AMP in the second incubation period, was not significant.

		Pretreatment with dibutyryl cyclic AMP for 60min	
	Control	+Dibutyryl cyclic AMP for next 20min	−Dibutyryl cyclic AMP for next 20min
Amylase release (% of control)	100±8	715±61*	693±50* (5)
Radioactivity in trichloroacetic acid precipitate (% of control)	100±5	150±13*	133±7* (5)
Radioactivity in amylase (% of control)	100±4	174±16**	150±5** (5)

Table 6. *Pulse-labelling experiments: effects of pretreatment of parotid tissue with adrenaline, phentolamine and propranolol before addition of [³H]leucine*

Results are means ± S.E.M. for parotid pieces preincubated for 30min in control Eagle's medium, incubated under the conditions shown for 60min and then pulsed under the same conditions for 20min with 20μCi of [³H]leucine. Results are expressed as % of control. Values in parentheses denote the numbers of observations. Control amylase release (linear over 80min) varied from 0.16 to 0.40 unit/60min per mg of tissue; control incorporation into trichloroacetic acid precipitates varied from 6966 to 12628d.p.m./mg of tissue and into amylase from 1894 to 4089d.p.m./mg of tissue. **$P<0.005$, *$P<0.02$ for difference from control.

		Radioactivity (% of control)	
Additions (μM)	Amylase release (% of control)	Trichloroacetic acid	Amylase
Control	100±15	100±4	100±4 (6)
Phentolamine (50)	125±14	87±6*	79±7* (4)
Adrenaline (20)	392±70**	46±5**	25±3** (4)
Adrenaline (20)+phentolamine (50)	513±67**	136±16	138±20 (6)
Control	100±8	100±3	100±3 (8)
Adrenaline (1)	199±14*	122±5*	123±8* (9)
Adrenaline (1)+propranolol (30)	108±9	111±2	100±5 (4)

significant. The adrenaline inhibition of [³H]leucine incorporation could also be reversed to control values by washing the tissue twice with control incubation medium after the first 60min and pulse labelling for 20min with [³H]leucine in control medium. Similarly, the inhibition of [³H]leucine incorporation by carbamoylcholine was reversed by washing out the carbamoylcholine in the same way. In some experiments a stimulation above control was observed, though the overall effect was not significant. [Incorporation into trichloroacetic acid-precipitable protein: control, 100±4% ($n=6$); carbamoylcholine, 131±16% ($n=6$). Incorporation into amylase: control, 100±5% ($n=6$); carbamoylcholine, 139±19% ($n=6$).]

A much lower concentration of adrenaline (1μM), which only had a 2-fold stimulatory effect on amylase release, increased [³H]leucine incorporation into trichloroacetic acid-precipitable protein and amylase by a small but significant extent ($P<0.02$), and this effect was blocked by propranolol (Table 6).

Discussion

The rat parotid gland has proved to be a good system for the study of the relationship between

release and biosynthesis of a secreted protein. The immunoprecipitation technique used for the extraction of radioactive amylase from the tissue in the present work was very specific and the incorporation rate of [^{3}H]leucine into amylase was linear with time over the time course of the experiments. The incorporation of [^{3}H]leucine into total protein and amylase appeared to represent synthesis *de novo*, since this was markedly decreased by puromycin, an inhibitor of protein synthesis. The amount of radioactivity incorporated into amylase was approx. 30% of that incorporated into total tissue protein, demonstrating that amylase represents a large percentage of the protein turnover of the rat parotid gland. No evidence was obtained for the existence of an immunologically related higher-molecular-weight precursor, although the methods used would probably not detect the transient presence of a precursor during transport of the enzyme into the lumen of the endoplasmic reticulum.

It is evident from the results that the rat parotid has α- and β-adrenergic receptors that may control both amylase release (see also Batzri *et al.*, 1971; Batzri & Selinger, 1973; Kanagasuntheram & Randle, 1976) and biosynthesis. However, adrenaline has a continuous stimulatory effect on amylase release over the same dose range as it has opposing effects on biosynthesis (Tables 1 and 6). In the case of release, the β-effect of adrenaline is stimulatory, whereas its α-effect does not appear to inhibit. Similarly at low concentrations or in the presence of phentolamine the β-effect of adrenaline increases amylase biosynthesis. However, at high concentrations the α-effect predominates causing inhibition. The β-regulatory effect on biosynthesis may be more relevant *in vivo*, since stimulation is observed at concentrations as low as 1μM-adrenaline. The α-effect of adrenaline may reflect a decreased uptake of amino acids into the cell (see also Grand & Gross, 1969), and this could be due to the decrease in tissue ATP concentration observed on pretreatment of parotid tissue with high concentrations (20μM) of adrenaline (Batzri & Selinger, 1973). Carbamoylcholine also inhibited [^{3}H]leucine incorporation into total protein and amylase without affecting the radioactivity of the amino acid pool. The inhibition by carbamoylcholine of amino acid incorporation into amylase in the exocrine pancreas is thought to be due to a decrease in tissue GTP concentration (Irwin & Tenenhouse, 1974).

It has been postulated that cyclic AMP may be involved in mediating the effects of β-adrenergic agents in the parotid gland, since these increase the tissue concentration of this nucleotide both *in vivo* (Guidotti *et al.*, 1972) and *in vitro* (Butcher *et al.*, 1976). The stimulatory effects of isoproterenol on amylase biosynthesis were mimicked by exogenous dibutyryl cyclic AMP. Stimulation by these agents was only observed on exposure of the tissue to effector before addition of radioactive leucine (compare Table 2 with Tables 4 and 5). Furthermore the effects were still observed even if the effector was absent during the subsequent pulse period with radioactive leucine (Table 5).

Thus these results suggest that an increase in amylase release is a pre-requisite for an increase in amylase biosynthesis. However, Grand & Gross (1969) observed that the further addition of adrenaline to a previously adrenaline-stimulated gland provoked a further increase in biosynthesis without a further increase in release, indicating that the coupling between synthesis and release is not complete. If a previous increase in release is essential for stimulation of biosynthesis, it should not be possible to dissociate these two processes. However, in endocrine tissues such as the islets of Langerhans it is possible to inhibit completely insulin release by omitting Ca^{2+} from the medium, although still observing stimulatory effects on biosynthesis (Lin & Haist, 1973). In exocrine tissues it is much more difficult to block release without affecting biosynthesis, since prolonged incubation of parotid tissue with Ca^{2+}-chelating agents is necessary to show inhibition of amylase release (Selinger & Naim, 1970; Batzri & Selinger, 1973; Dormer & Ashcroft, 1974), and this treatment undoubtedly has other metabolic effects.

Insulin increased [^{3}H]leucine incorporation into total tissue protein and amylase without stimulating amylase release, and was the only stimulator of incorporation that did not have to be added to the tissue before [^{3}H]leucine to have an effect. This effect, seen at approximately physiological concentrations, may be due to an increased amino acid uptake by the tissue. Insulin is known to increase amino acid uptake in other tissues such as muscle (Narahara & Holloszy, 1974).

Thus, although amylase release and biosynthesis in the rat parotid appear to be closely linked, it is not clear whether an increase in biosynthesis is dependent on previous stimulation of release or occurs via an independent parallel mechanism. These studies show that the presence of a variety of methods for modulating synthesis of a specific secreted polypeptide in this system make it an excellent model in which to investigate further the mechanisms involved.

We thank members of the Department for much helpful discussion and the British Diabetic Association for financial support of these studies.

References

Ball, W. D. (1974) *Biochim. Biophys. Acta* **341**, 305–317
Batzri, S. & Selinger, Z. (1973) *J. Biol. Chem.* **248**, 356–360
Batzri, S., Selinger, Z. & Schramm, M. (1971) *Science* **174**, 1029–1031

Bdolah, A., Ben-Zvi, R. & Schramm, M. (1964) *Arch. Biochem. Biophys.* **104**, 58–66
Bernfeld, P. (1955) *Methods Enzymol.* **1**, 149–158
Butcher, F. R., McBride, P. A. & Rudich, L. (1976) *Mol. Cell. Endocrinol.* **5**, 243–254
Campbell, P. N. & Blobel, G. (1976) *FEBS Lett.* **72**, 215–226
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Dormer, R. L. & Ashcroft, S. J. H. (1974) *Biochem. J.* **144**, 543–550
Grand, R. J. & Gross, P. R. (1969) *J. Biol. Chem.* **244**, 5608–5615
Guidotti, A., Weiss, B. & Costa, E. (1972) *Mol. Pharmacol.* **8**, 521–530
Gurvich, A. E., Kuzovleva, O. B. & Tumanova, A. E. (1961) *Biokhimiya* **25**, 803–809
Hales, C. N. & Randle, P. J. (1963) *Biochem. J.* **88**, 137–145
Irwin, C. & Tenenhouse, A. (1974) *Can. J. Physiol. Pharmacol.* **52**, 632–640
Kanagasuntheram, P. & Randle, P. J. (1976) *Biochem. J.* **160**, 547–564
Lin, B. J. & Haist, R. E. (1973) *Endocrinology* **92**, 735–742
Lin, B. J. & Haist, R. E. (1975) *Endocrinology* **96**, 1247–1253
Lowry, O. H., Rosebrough, N. J., Farr, A. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Messer, M. & Dean, R. T. (1975) *Biochem. J.* **151**, 17–22
Miles, L. E. M. & Hales, C. N. (1968) *Biochem. J.* **108**, 611–618
Narahara, H. T. & Holloszy, J. O. (1974) *J. Biol. Chem.* **249**, 5435–5443
Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6334
Rosenfeld, M. G., Abrass, T. B. & Chang, B. (1976) *Endocrinology* **99**, 611–618
Schneyer, C. A. & Hall, H. D. (1966) *Proc. Soc. Exp. Biol. Med.* **121**, 96–100
Schramm, M. & Selinger, Z. (1975) *J. Cyclic Nucleotide Res.* **1**, 181–192
Selinger, Z. & Naim, E. (1970) *Biochim. Biophys. Acta* **203**, 335–337
Taylor, K. W. & Parry, D. G. (1967) *J. Endocrinol.* **39**, 457–458
Taylor, K. W., Parry, D. G. & Smith, G. H. (1964) *Nature (London)* **203**, 1144–1145