Effects of Proteinase Inhibitors on Adenylate Cyclase

Patrick J. McILROY, Nancy D. RICHERT* and Robert J. RYAN

Department of Cell Biology, Mayo Medical School and Foundation, Rochester, MN 55901, U.S.A.

(Received 15 October 1979)

The effects of a number of proteinase inhibitors on rat ovarian and rat hepatic adenylate cyclase preparations were examined. *N* α -tosylarginine methyl ester, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one, 1-chloro-4-methyl-3-L-tosylamidopentan-2-one and other low-molecular-weight proteinase inhibitors blocked hormonally stimulated adenylate cyclase from either source with hepatic preparations requiring higher concentrations. Addition of nucleotides (ATP, GTP, GDP, CTP or ITP) to inhibited ovarian preparations did not reverse inhibition, nor did dithiothreitol reverse phenylmethanesulphonyl fluoride-inhibited ovarian adenylate cyclase. The kinetics of the inhibition of rat ovarian adenylate cyclase were examined by following the production of cyclic AMP after the addition of inhibitors to membrane preparations preincubated under assay conditions with human chorionadotropin, guanosine 5'-[β -imido]triphosphate or NaF. 7-Amino-1-chloro-3-L-tosylamidoheptan-2-one, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one and 1-chloro-4-methyl-3-L-tosylamidopentan-2-one had two effects on human-chorionadotropin-stimulated adenylate cyclase. At low concentrations (≤ 0.2 mM) there was an irreversible inhibition of hormonally-stimulated cyclase with maximum first-order inhibitory rate constants of 0.05–0.08 min⁻¹. At higher concentrations the irreversible effect persisted, but, in addition, there was a marked decrease in the cyclase initial velocity to 25–50% of that of control values. *N* α -tosylarginine methyl ester had similar effects; at low concentrations (≤ 2 mM) it inhibited irreversibly, and at higher concentrations it decreased the initial velocity (50% at 10 mM). At high concentrations (> 3 mM) *N* α -tosylarginine methyl ester also inhibited NaF- and guanosine 5'-[β -imido]-triphosphate-stimulated cyclase but in a reversible manner. 7-Amino-1-chloro-3-L-tosylamidoheptan-2-one inhibited NaF-stimulated adenylate cyclase in two ways, as for human-chorionadotropin-stimulated adenylate cyclase, but required 10–20-fold higher concentrations. The low-concentration irreversible effect can be explained by a continual inactive $\rightleftharpoons$ active conversion of adenylate cyclase during hormonal stimulation in which the inactive to active conversion is blocked by the inhibitors. The high-concentration effect is a direct one on the active catalytic moiety of the enzyme.

Abbreviations used: Dns, 5-dimethylaminonaphthalene-1-sulphonyl; Suc, succinyl; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; Leu-NNap, L-leucine naphthylamide; Tos-Arg-OMe, *N* α -tosyl-arginine methyl ester; Tos-Lys-OMe, *N* α -tosyl-lysine methyl ester; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('tosylphenylalanine chloromethyl ketone'); Tos-Lys-CH₂Cl, 1-chloro-4-methyl-3-L-tosylamidopentan-2-one ('tosyl-lysine chloromethyl ketone'); Tos-Leu-CH₂Cl, 1-chloro-4-methyl-3-L-tosylamidopentan-2-one ('tosyl-leucine chloromethyl ketone'); Bz, benzoyl. Abbreviations for other peptide derivatives follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J. (1972) 126, 773–780].

Recently, there have been a number of reports describing the stimulation of adenylate cyclase (EC 4.6.1.1) by serine proteinases both in intact (Ryan *et al.*, 1975; Guiraud-Simplot & Golobert, 1977; Richert *et al.*, 1978) and broken-cell preparations (Hanoune *et al.*, 1977; Richert & Ryan, 1977a; Richert *et al.*, 1978; Anderson *et al.*, 1978). Investigations of the mechanism of proteinase stimulation, with inhibitors of differing specificities and modes of action, showed that a number of

* Present address: National Cancer Institute, Bethesda, MD 20014, U.S.A.

low-molecular-weight serine proteinase inhibitors could decrease hormonal stimulation of rat ovarian adenylate cyclase (Richert & Ryan, 1977b; Richert *et al.*, 1978). These findings led to the suggestion that endogenous plasma-membrane proteinases may be involved in hormonal stimulation of adenylate cyclase (Richert & Ryan, 1977b; Richert *et al.*, 1978). The present paper gives data that extend observations on the effects of low-molecular-weight inhibitors on the glucagon-stimulated adenylate cyclase of rat liver and provides further support for the involvement of proteinases in hormonal stimulation of ovarian adenylate cyclase. A preliminary report of part of this work has been presented elsewhere (McIlroy & Ryan, 1978).

Materials and Methods

Highly purified human choriogonadotropin, CR-121, was a gift from the National Institute of Child Health and Human Development and from Dr. Robert Canfield, Columbia University, New York, NY 10032, U.S.A. Na¹²⁵I (carrier-free) was from The Radiochemical Centre, and [α -³²P]ATP (10–30 Ci/mmol), cyclic [³H]AMP (30–50 Ci/mmol) and ¹²⁵I-labelled glucagon were from New England Nuclear. Dns-Glu-Gly-Arg-CH₂Cl was kindly provided by Dr. Elliot Shaw, Brookhaven National Laboratory, Upton, NY 11473, U.S.A., Dns-Arg-4-ethylpiperidineamide by Dr. Kenneth Mann, Mayo Clinic, Rochester, MN 55901, U.S.A., MeO-Suc-Ala₂-Pro-Val-CH₂Cl by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA 30332, U.S.A., and chymostatin, antipain and leupeptin by Dr. Walter Troll, New York University Medical Center, New York, NY 10016, U.S.A. Dns-Leu-NNap and Dns-Leu-CH₂Cl were synthesized by Dr. Ronald Whitley, Mayo Clinic, Rochester, MN 55901, U.S.A. H-Thr-Pro-Arg-Lys-OH was obtained from Beckman, pancreatic trypsin inhibitor, lima-bean trypsin inhibitor and trypsin were from Worthington, and all other enzymes and inhibitors from either Chemical Dynamics, South Plainfield, NJ 07080, U.S.A., or Sigma. Other reagents were of reagent grade.

Preparation of 2000 g pellets

Preparation of the membrane-enriched 2000 g pellet fraction from superovulated rat ovaries, 7–10 days after priming with pregnant mare's serum gonadotropin and human choriogonadotropin, was performed as previously described (Lee & Ryan, 1973). A similar hepatic fraction was prepared from livers of immature fed female rats.

Adenylate cyclase assays

Adenylate cyclase activity was determined by the method of Birnbaumer *et al.* (1976) with modi-

fications as previously described (Lee, 1976). The incubation mixture contained 25 mM-Tris/HCl, pH 7.0, 3 mM-MgCl₂, 1 mM-EDTA, 1 mM-3-isobutyl-1-methylxanthine, 1 mM-cyclic AMP, 1 mM-[α -³²P]ATP (30–50 c.p.m./pmol), an ATP-regenerating system consisting of 20 mM-phosphocreatine and 0.2 mg of creatine phosphokinase/ml, and additions as indicated in a final volume of 50 μ l. NaF (20 mM), human choriogonadotropin (1 μ g/ml) and most inhibitors were dissolved and diluted in 40 mM-Tris/HCl buffer (pH 7.4). Glucagon (1 μ M) and some inhibitors (see the Tables) were dissolved and diluted in 1 mM-HCl before addition to the assays. This did not change the pH of the assay mixture. Other inhibitors (see the Tables) were dissolved and diluted in methanol before addition to the assays. The final concentration of methanol in the assay mixture was 10%. [Methanol increases adenylate cyclase stimulability by human choriogonadotropin at concentrations of up to 12% (v/v) and is inhibitory at higher concentrations. Abramowitz & Birnbaumer (1979) found that ethanol gave similar results.] Appropriate solvent controls were included in each assay. Unless otherwise noted, incubations were initiated by the addition of the membrane preparation (equivalent to 1 mg wet wt. of tissue) and were carried out for 20 min at 30°C. The reaction was terminated by the addition of 100 μ l of 40 mM-ATP, 12.5 mM-cyclic AMP, cyclic [³H]AMP (approximately 10000 c.p.m.) and 1% (w/v) sodium dodecyl sulphate followed by heating at 100°C for 3 min. Cyclic AMP was isolated by chromatography on Dowex and alumina as described by Lee (1976). The eluates from the alumina column were counted for radioactivity in 9 ml of 3a70B scintillation fluid (Research Products International Corp., Elk Grove Village, IL 60007, U.S.A.) in a Searle liquid-scintillation counter. Adenylate cyclase activity was expressed as pmol of cyclic AMP formed/20 min per mg of protein. Assays were routinely run in duplicate or triplicate, and an S.E.M. of ± 5 –10% was found. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Binding of ¹²⁵I-labelled hormones to 2000 g pellets

¹²⁵I-labelled human choriogonadotropin preparation and binding to ovarian membrane preparations (2000 g) were performed as previously described (Lee & Ryan, 1973). Human choriogonadotropin was labelled by a modification (Lee & Ryan, 1973) of the method of Greenwood *et al.* (1963). Unless otherwise noted, binding assays were performed in 40 mM-Tris/HCl buffer, pH 7.4, containing 0.1% bovine serum albumin and 1 ng of ¹²⁵I-labelled human choriogonadotropin in a final volume of 1 ml. The incubations were initiated by the addition of membrane preparation (equivalent to

Table 1. *Effect of proteinase inhibitors on adenylate cyclase activity and hormone binding in rat ovarian and liver membrane preparations*
Adenylate cyclase assays and ¹²⁵I-labelled hormone binding assays were performed as described in the Materials and Methods section in the presence of increasing concentrations of inhibitors up to the maximum dose indicated. Adenylate cyclase results are expressed as the dose of inhibitor necessary to decrease enzyme activity to 50% of appropriate control activity (ID₅₀). ¹²⁵I-labelled human chorionadotropin binding assays were incubated at 37°C for 1 h. Binding of ¹²⁵I-labelled hormone, at the maximum inhibitor dose, is expressed as % of control binding in the absence of inhibitors. Abbreviation: NT, not tested.

Inhibitor	Solvent	Max. dose (mM)	Ovary				Liver			
			ID ₅₀			Human ¹²⁵ I-choriogonadotropin binding (% of control)	ID ₅₀			¹²⁵ I-Glucagon binding (% of control)
			Basal	+NaF	+Human chorio-gonadotropin		Basal	+NaF	+Glucagon	
Irreversible										
Phenylmethanesulphonyl fluoride	Methanol	5	3.0	3.5	0.8	56*	†	100.0‡	50.0‡	79§
Tos-Lys-CH ₂ Cl	Water	5	0.8	1.0	0.4	111	1.0	2.0	0.4	58*
Tos-Phe-CH ₂ Cl	Methanol	10	3.0	3.0	0.1	107	†	†	0.5	75*
Tos-Leu-CH ₂ Cl	Methanol	2	0.6	3.0‡	0.1	44*	2.0	2.0	0.4	114
Di-isopropyl phosphorofluoridate	Water	30	†	70.0‡	4.0	NT				
Competitive										
Tos-Arg-OMe	Water	10	10.0	40.0‡	2.0	94	30.0	90.0‡	7.0	0§
Tos-Lys-OMe	Water	30	10.0	†	4.0	80	15.0	†	15.0	30§
Leu-NNap	HCl	2	1.0	2.0	0.2	10*	1.0	2.5	0.8	0§

* No inhibition of ¹²⁵I-labelled hormone binding at doses necessary for effective adenylate cyclase inhibition.
† No effect at highest dose tested.
‡ Partial inhibition at maximum inhibitor dose. Value in Table is extrapolated.
§ Inhibition of binding parallels inhibition of adenylate cyclase.

2.5 mg wet wt. of ovary) and were carried out as indicated.

Binding of ^{125}I -labelled glucagon to 2000 g hepatic membrane preparations was measured essentially as described by Lad *et al.* (1977); membranes (2000 g pellet) equivalent to 2.5 mg wet wt. of tissue were incubated for 10 min at 30°C with 1 nM- ^{125}I -labelled glucagon, 20 mM-Tris/HCl, pH 7.0, 0.2% bovine serum albumin, 1 mM-dithiothreitol and 30 μM -adenosine 5'-[$\beta\gamma$ -imido]triphosphate in a final volume of 250 μl . The membranes were collected by filtration on prewashed [$2 \times 1\text{ ml}$ of 0.1% bovine serum albumin/20 mM-Tris/HCl (pH 7.0)/1 mM-dithiothreitol] 0.45 μm Metrice membrane filters (Gelman Instruments Co., Ann Arbor, MI 48106, U.S.A.). The tubes and filters were rinsed [$2 \times 1\text{ ml}$ of 0.1% bovine serum albumin/20 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol], and the bound ^{125}I -labelled glucagon was determined by counting in a Packard Autogamma spectrometer.

In both the ^{125}I -labelled human choriogonadotropin and ^{125}I -labelled glucagon binding assays correction was made for non-specific binding by inclusion of controls containing an excess of unlabelled hormone.

Results

Inhibition of adenylate cyclase by serine proteinase inhibitors

Table 1 shows the effects of a variety of serine proteinase inhibitors, both irreversible and reversible, on rat ovarian and hepatic adenylate cyclase. The concentrations of inhibitors necessary to affect enzyme activity were dependent upon the specificities and mode of action of the inhibitor and the source of the adenylate cyclase preparation used.

Tos-Phe-CH₂Cl, an irreversible inhibitor and active-site titrant of α -chymotrypsin (Shaw, 1970), and Tos-Leu-CH₂Cl, an irreversible inhibitor of chymotrypsin (Tobita & Folk, 1967; Shaw, 1970)

were more effective on ovarian than on hepatic preparations. Tos-Lys-CH₂Cl, an irreversible inhibitor of trypsin (Shaw, 1970; Shaw & Glover, 1970), was equally effective on both hepatic and ovarian adenylate cyclase. A comparison of arginine and lysine derivatives revealed that the arginine derivative, Tos-Arg-OMe, was more effective in both hepatic and ovarian preparations than its lysine counterpart, Tos-Lys-OMe.

In addition to the above proteinase inhibitors, two reagents known to act on reactive serine moieties, phenylmethanesulphonyl fluoride and di-isopropyl phosphorofluoridate (Shaw, 1970; Barrett, 1977a), were examined, and both were found to inhibit ovarian adenylate cyclase. Hepatic adenylate cyclase was slightly inhibited by phenylmethanesulphonyl fluoride at the concentrations tested. The effect of di-isopropyl phosphorofluoridate on the liver enzyme was not examined.

The effectiveness of Leu-NNap, a competitive inhibitor of arylamidase (Barrett, 1977a), was similar to that of Tos-Leu-CH₂Cl, being more effective on ovarian than on hepatic preparations.

It appears that the proteinase inhibitors do not act on ovarian cyclase by inhibiting the binding of human choriogonadotropin. There was very little effect of the inhibitors on ^{125}I -labelled human choriogonadotropin binding to ovarian pellets when measured after incubation for 1 h at 37°C at concentrations that effectively inhibited adenylate cyclase (Table 1). However, inhibition of glucagon-stimulated adenylate cyclase by phenylmethanesulphonyl fluoride, Tos-Arg-OMe, Tos-Lys-OMe and Leu-NNap could be accounted for by the effect of these inhibitors on binding (Table 1). The effect of the other inhibitors on glucagon binding, Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl and Tos-Leu-CH₂Cl, was not sufficient to account for their inhibitory action on glucagon-stimulated adenylate cyclase (Table 1).

The possibility that the above low-molecular-weight inhibitors were acting by some mechanism

Table 2. *Effect of control compounds on adenylate cyclase activity in rat ovarian membrane preparations*
Adenylate cyclase assays were performed as described in the Materials and Methods section with the additions indicated.

Inhibitor	Solvent	Dose (mM)	Adenylate cyclase (pmol of cyclic AMP/20 min per mg of protein)		
			Basal	+NaF	+Human choriogonadotropin
None	—	—	58	557	226
Toluene	—	10	63	519	321
Toluenesulphonic acid	Water	10	69	492	220
None	—	—	22	573	86
Tos-Arg	HCl	10	17	578	63
Tos-Lys	HCl	3	21	503	78

Table 3. *Effect of proteinase inhibitors on adenylate cyclase activity and hormone binding in rat ovarian membrane preparations*

Adenylate cyclase assays and ^{125}I -labelled hormone binding assays were performed as described in the Materials and Methods section in the absence and presence of increasing concentrations of inhibitors up to the maximum dose indicated. Adenylate cyclase results are expressed as that dose of inhibitor necessary to decrease enzyme activity to 50% of the appropriate control activity. ^{125}I -labelled human choriogonadotropin binding assays were performed under the conditions of the adenylate cyclase assay and were incubated at 30°C for 20 min. Binding is expressed as % of control binding of ^{125}I -labelled human choriogonadotropin at an inhibitor dose of $3 \times \text{ID}_{50}$ for human choriogonadotropin-stimulated cyclase. Abbreviation: NT, not tested.

Inhibitor	Solvent	Max. concn. (mM)	ID ₅₀			Human ^{125}I -labelled choriogonadotropin binding (% of control)
			Basal	+NaF	+Human choriogonadotropin	
Dns-Glu-Gly-Arg-CH ₂ Cl	HCl	1.0	0.7	1.0	0.08	89
Dns-Arg-4-ethylpiperidineamide	Methanol	1.0	0.7	0.9	0.15	15
Pancreatic trypsin inhibitor	Water	0.15	*	*	0.3†	NT
Dns-Leu-NNap	Methanol	1.0	1.0	1.0	0.4	110
Chymostatin	HCl	1.0	10.0†	*	0.6	NT
MeO-Suc-Ala-Pro-Val-CH ₂ Cl	Methanol	3.0	5.0†	*	1.5	91
Chloroquine	Water	3.0	5.0†	3.0	1.7	67
N-Ac-Val	Water	30.0	4.0	2.5	2.0	75
Dns-Leu-CH ₂ Cl	Methanol	3.0	*	*	2.0	71
N-Ac-Glu	Water	25.0	2.5	3.0	2.5	67
N-Ac-Tyr	Water	50.0	3.5	3.5	3.0	83
Antipain	Water	10.0	6.0	30.0†	3.0	76
1,7-Phenanthroline	Methanol	3.0	*	*	3.0	63
N-Ac-Ser	Water	100.0	3.5	4.5	3.5	82
N-Cbz-L-Lys-OMe	Water	100.0	50.0	200.0†	5.0	80
Leupeptin	Water	5.0	20.0†	*	5.0	63
1,10-Phenanthroline	Methanol	3.0	*	*	5.0†	51
N-Bz-L-Arg-OMe	Water	10.0	*	40.0†	6.0	NT
Lima-bean trypsin inhibitor	Water	0.1	*	*	7.0†	45
Leu-Trp	Methanol	10.0	NT	NT	15.0†	NT
N-Ac-D-Phe-OMe	Methanol	100.0	600.0†	400.0†	20.0	52
Benzamidine	Water	100.0	100.0	100.0	25.0	105
N-Ac-L-Gly-OMe	Water	100.0	50.0	40.0	30.0	66
N-Ac-L-Phe-OMe	Methanol	100.0	120.0†	300.0†	30.0	44
N-Ac-D-Trp-OMe	Methanol	30.0	*	*	30.0	77
N-Ac-L-Trp-OMe	Methanol	30.0	*	*	30.0	52
N-Ac-L-Tyr-OEt	Water	10.0	30.0†	100.0†	30.0†	56
N-Bz-L-Ala-OMe	Methanol	100.0	130.0†	70.0	30.0	17
N-Ac-L-Ala-OMe	Water	100.0	65.0	200.0†	35.0	65
N-Ac-L-Gly-Lys-OMe	Water	100.0	75.0	100.0	35.0	70
N-Ac-L-Arg-OMe	Water	100.0	400.0†	*	40.0	52
N-Ac-L-Met-OMe	Water	100.0	40.0	50.0	40.0	56
N-Ac-L-Leu-OMe	Methanol	100.0	*	*	70.0	58

* No effect at maximum dose tested.

† Partial inhibition was seen at maximum dose; the value in the Table was obtained by extrapolation.

other than through their known effects on proteinases was examined in a variety of ways. Table 2 shows the effect of control incubations on ovarian adenylate cyclase. Neither toluene nor toluene sulphonic acid, which comprise part of the structure of a large number of the inhibitors, have any effect on adenylate cyclase activity at concentrations that are effective for the inhibitors. Similarly, the tosylated amino acids N^α -tosyl-L-arginine and N^α -tosyl-

L-lysine had no inhibitory action on basal or stimulated (hormone or NaF) adenylate cyclase (Table 2), whereas their methyl ester and chloromethyl ketone derivatives did (Table 1).

Further investigations of the effects of a variety of compounds, in addition to those in Tables 1 and 2, were undertaken to obtain support for the role of proteinases in the hormonal stimulation of rat ovarian adenylate cyclase. Table 3 lists in order of

Table 4. Compounds found ineffective in inhibiting rat ovarian adenylate cyclase

Adenylate cyclase assays were performed as described in the Materials and Methods section with the addition of compounds over the indicated dose ranges.

Compound	Concentration (M)	
	Min.	Max.
Ascorbic acid	10^{-4}	10^{-2}
NH ₄ Cl	10^{-3}	10^{-2}
Colchicine	10^{-8}	10^{-5}
Ouabain	10^{-8}	10^{-4}
Phorbol ester	10^{-8}	10^{-4}
Retinoic acid	10^{-8}	10^{-6}
H-Thr-Pro-Arg-Lys-OH	10^{-5}	10^{-3}
Vinblastine	10^{-8}	10^{-5}

decreasing potency those compounds that inhibited human-choriogonadotropin-stimulated ovarian adenylate cyclase. Effects on binding were also examined. Most of the compounds had some effect on binding, but generally these effects were not sufficient to explain their inhibitory action, the exceptions being Dns-Arg-4-ethylpiperidineamide and *N*-Bz-Ala-OMe. In contrast, the compounds listed in Table 4 had neither an inhibitory nor a stimulatory effect on ovarian adenylate cyclase at the concentrations shown.

Effect of dithiothreitol on adenylate cyclase inhibition

In addition to their actions on active serine and histidine groups, phenylmethanesulphonyl fluoride and chloromethyl ketones attack free thiol groups (Whitaker & Perez-Villasenor, 1967; Rossman *et al.*, 1974). In the case of phenylmethanesulphonyl fluoride, this attack is reversed by the addition of dithiothreitol (Whitaker & Perez-Villasenor, 1967). In order to investigate the possibility that some of the proteinase inhibitors listed in Table 1 were acting through the removal of a necessary thiol group, the ability of dithiothreitol to reverse inhibition of ovarian adenylate cyclase was examined. Dithiothreitol was added to human-choriogonadotropin-stimulated adenylate cyclase incubations already inhibited by phenylmethanesulphonyl fluoride, and its ability to reverse the inhibition was assessed by comparing cyclic AMP production with that of control incubations. Fig. 1 shows that 3 mM-dithiothreitol had little, if any, effect on inhibition of adenylate cyclase by 1 or 3 mM-phenylmethanesulphonyl fluoride.

Effect of nucleotides on adenylate cyclase inhibition

Similar experiments were conducted to examine the possibility that the inhibitors were acting by

Fig. 1. Effect of dithiothreitol on phenylmethanesulphonyl fluoride-inhibited human choriogonadotropin-stimulated rat ovarian adenylate cyclase

Adenylate cyclase incubations stimulated by human choriogonadotropin were incubated as described in the Materials and Methods section. After 10 min, sufficient concentrated phenylmethanesulphonyl fluoride (10 mM and 30 mM) was added to raise the concentration to 1 and 3 mM; 10 min after the addition of the phenylmethanesulphonyl fluoride, sufficient concentrated dithiothreitol (30 mM) was added to raise its concentration to 3 mM. The incubations were terminated as described in the Materials and Methods section at the times shown. Control incubations contained the appropriate solvent. Each point is the average of triplicate incubations. ○, ●, human choriogonadotropin control; △, ▲, 1 mM-; □, ■, 3 mM-phenylmethanesulphonyl fluoride at 10 min; ●, ▲, ■, 3 mM-dithiothreitol at 20 min.

altering nucleotide concentrations in the assay. The addition of 1 mM-ATP or GTP to ovarian preparations already inhibited by 10 mM-Tos-Arg-OMe (Fig. 2a) or Tos-Phe-CH₂Cl (Fig. 2b) had no effect on cyclic AMP production. Other nucleotides, GDP, ITP, CTP and p[NH]ppG, were tried and, except for

Fig. 2. Effect of GTP and ATP on Tos-Arg-OMe- and Tos-Phe-CH₂Cl-inhibited human choriogonadotropin-stimulated rat ovarian adenylate cyclase

Adenylate cyclase incubations were treated as described in Fig. 1, with the following exceptions. The inhibitors added were 10mM-Tos-Arg-OMe and 5mM-Tos-Phe-CH₂Cl; 20min after the addition of the inhibitors, sufficient concentrated GTP (10mM) and ATP (10mM) were added to raise the concentrations to 1mM. Each point is the average of duplicate determinations. ○, ●, Human choriogonadotropin controls; △, ▲, 10mM-Tos-Arg-OMe, □, ■, 5mM-Tos-Phe-CH₂Cl added at the indicated times (a) ●, ▲, ■, 1mM-GTP added 20min after inhibitors; (b) ●, ▲, ■, 1mM-ATP added 20min after inhibitors.

p[NH]ppG, were found to be ineffective in restoring adenylate cyclase activity. As is shown in Fig. 3a, the addition of 0.1mM-p[NH]ppG stimulated adenylate cyclase activity in the presence of 10mM-Tos-Arg-OMe; however, the resultant increase in enzyme activity was less than that which occurred when p[NH]ppG was added in the absence of 10mM-Tos-Arg-OMe. In the reverse experiment (Fig. 3b), the addition of 10mM-Tos-Arg-OMe to adenylate cyclase preparations previously stimulated with 0.1mM-p[NH]ppG caused a marked inhibition of enzyme activity, by approximately the same extent as in the previous experiment.

Kinetics of inhibition

Chloromethyl ketones inactivate serine proteinases irreversibly by alkylating active-site histidine residues (Shaw, 1970). If the chloromethyl ketones inhibit adenylate cyclase through a similar mechanism, then the inhibition should follow ir-

reversible kinetics. Irreversible inactivation can be followed by measuring the production of a product with respect to time in the presence of the inhibitor. The production of product is described by the equation:

$$\frac{V_0}{k_1} (1 - e^{-k_1 t}) = K_m^a \ln \left(\frac{a_0}{a_0 - [\text{cyclic AMP}]} \right) \quad (1)$$

where V_0 is the initial velocity, k_1 the first-order inhibitory rate constant, t the time in minutes, a_0 the initial substrate concentration and K_m^a its Michaelis constant (Laidler & Bunting, 1973).

In the case of the adenylate cyclase assay the substrate concentration was kept constant by the presence of an ATP-regenerating system and eqn. (1) can be reduced to:

$$\frac{V_0}{k_1} (1 - e^{-k_1 t}) = [\text{cyclic AMP}] \quad (2)$$

Fig. 3. Effect of $p[\text{NH}]ppG$ and Tos-Arg-OMe on human choriogonadotropin-stimulated rat ovarian adenylate cyclase. Adenylate cyclase incubations were treated in a similar manner to those in Fig. 1. (a) Sufficient concentrated Tos-Arg-OMe (100 mM) was added at 10 min to human choriogonadotropin-stimulated cyclase incubations to raise its final concentrations to 10 mM; 10 min later sufficient $p[\text{NH}]ppG$ (1 mM) was added to raise its final concentration to 0.1 mM. (b) The order of addition was reversed; 0.1 mM- $p[\text{NH}]ppG$ was present in the human choriogonadotropin-stimulated cyclase incubations at the start, and Tos-Arg-OMe was added 10 min later. Each point is the average of duplicate determinations. (a) $\circ$, Human choriogonadotropin control; $\triangle$, $\blacktriangle$, 10 mM- Tos-Arg-OMe ; $\bullet$, $\blacktriangle$, 0.1 mM- $p[\text{NH}]ppG$. (b) $\circ$, human choriogonadotropin + $p[\text{NH}]ppG$ control; $\bullet$, 10 mM- Tos-Arg-OMe .

This equation can be rearranged and linearized:

$$\ln\left(\frac{V_0}{k_i} - [\text{cyclic AMP}]\right) = k_i t + \ln \frac{V_0}{k_i} \quad (3)$$

With the aid of a Wang 2000 computer, data from experiments similar to those previously described (inhibitors were added to human choriogonadotropin-stimulated adenylate cyclase incubations and the production of cyclic AMP with respect to time was followed) were fitted to eqn. (3), and the initial adenylate cyclase velocity (V_0) and pseudo-first-order inhibitory rate constant (k_i) were obtained. The data for increasing concentrations of Tos-Arg-OMe , $\text{Tos-Lys-CH}_2\text{Cl}$, $\text{Tos-Phe-CH}_2\text{Cl}$, $\text{Tos-Leu-CH}_2\text{Cl}$ and Leu-NNap are shown in Table 5. As expected, inhibition by $\text{Tos-Lys-CH}_2\text{Cl}$, $\text{Tos-Phe-CH}_2\text{Cl}$ and $\text{Tos-Leu-CH}_2\text{Cl}$ appeared to be irreversible, since the inhibitory rate constants (k_i)

for each compound increased with increasing concentrations of the inhibitors. Surprisingly, it appeared that Tos-Arg-OMe also inhibited irreversibly. As is shown in Table 5, Tos-Arg-OMe had a dual effect; at low concentrations it inhibited irreversibly and at high concentrations, in addition to acting irreversibly, it also affected the initial velocity.

A similar dual effect was also observed for other proteinase inhibitors. $\text{Tos-Phe-CH}_2\text{Cl}$, $\text{Tos-Lys-CH}_2\text{Cl}$ and $\text{Tos-Leu-CH}_2\text{Cl}$ inhibited irreversibly at low concentrations and also decreased the initial velocity at high concentrations (Table 5).

The inhibitory effect of Leu-NNap appeared to be that of a reversible inhibitor, although the data are inconclusive. Increasing concentrations of the compound had a pronounced effect on the initial velocity of human choriogonadotropin-stimulated adenylate cyclase when added to the incubations and only a slight effect on the inhibitory rate constant (Table 5).

Table 5. Initial velocities and first-order inhibitory rate constants for proteinase inhibitors in human choriogonadotropin-stimulated cyclase incubations

Adenylate cyclase assays were performed as described in Fig. 1. The indicated concentrations of inhibitors were added to human choriogonadotropin-stimulated adenylate cyclase incubations after 10 min at 30°C. Incubations were stopped at 10, 20, 30, 40 and 50 min. Control incubations received portions of the appropriate solvent. The data obtained were fitted to eqn. (3) with the aid of a Wang 2000 computer and the initial velocity (V_0) and first-order inhibitory rate constant (k_i) for each curve obtained.

Inhibitor	Concentration (mM)	V_0 (pmol/min per mg)	k_i (min^{-1})
Tos-Arg-OMe	Control	21.0	0.007
	1.0	17.8	0.015
	2.0	23.4	0.047
	5.0	15.3	0.048
	10.0	9.8	0.050
Tos-Phe-CH ₂ Cl	Control	36.7	0.008
	0.05	29.6	0.017
	0.2	11.6	0.032
	0.5	8.6	0.052
	1.0	8.0	0.053
Tos-Lys-CH ₂ Cl	Control	52.5	0.002
	0.1	55.0	0.037
	0.2	52.9	0.064
	0.5	34.1	0.083
	1.0	23.9	0.075
Tos-Leu-CH ₂ Cl	Control	49.2	0.014
	0.025	60.2	0.037
	0.05	70.1	0.062
	0.1	55.6	0.060
	0.2	36.2	0.060
	0.5	19.2	0.052
Leu-NNap	Control	43.8	0.007
	0.025	61.9	0.034
	0.05	44.7	0.011
	0.1	33.4	0.006
	0.2	31.6	0.019
	0.5	18.5	0.024

The second effect of Tos-Arg-OMe and the chloromethyl ketone derivatives suggested a direct action on the catalytic adenylate cyclase moiety. This view is supported by the data in Fig. 3 in which Tos-Arg-OMe inhibited p[NH]ppG-stimulated adenylate cyclase but in a reversible manner. To verify the above, the kinetics of inhibition of NaF-stimulated adenylate cyclase by Tos-Arg-OMe and Tos-Lys-CH₂Cl were examined. Table 6 shows that Tos-Arg-OMe inhibited NaF-stimulated adenylate cyclase but in a reversible manner. Substrate-velocity studies of NaF-stimulated adenylate cyclase

Table 6. Initial velocities and first-order inhibitory rate constants for inhibition of NaF-stimulated adenylate cyclase by Tos-Arg-OMe and Tos-Lys-CH₂Cl

Adenylate cyclase assays and data analysis were performed as in Table 5 except for the presence of NaF instead of human choriogonadotropin as the stimulatory agent.

Inhibitor	Concentration (mM)	V_0 (pmol/min per mg)	k_i (min^{-1})
Tos-Arg-OMe	Control	161	0.013
	1.0	158	0.020
	3.0	127	0.022
	10.0	85	0.008
Tos-Lys-CH ₂ Cl	Control	161	0.013
	0.4	131	0.024
	2.0	118	0.028
	10.0	36	0.057

in the absence and presence of 10 mM-Tos-Arg-OMe showed only an effect on the maximal velocity, with no change in K_m or Hill coefficient. Similar results were also obtained for human-choriogonadotropin-stimulated and basal adenylate cyclase in the absence and presence of 2 and 5 mM-Tos-Arg-OMe (results not shown). Tos-Lys-CH₂Cl also inhibited NaF-stimulated adenylate cyclase, but it acted in an irreversible manner and at higher doses than required for the irreversible inhibition of hormonally stimulated adenylate cyclase (Table 6).

Reversal of Tos-Arg-OMe inhibition

The fact that Tos-Arg-OMe, a competitive proteinase inhibitor, irreversibly inhibited hormonally stimulated adenylate cyclase was surprising, and attempts were made to reverse its inhibitory action by removing the compound by proteolytic breakdown or by washing Tos-Arg-OMe-treated 2000g membrane preparations. The inclusion of sufficient trypsin to stimulate maximally rat ovarian adenylate cyclase (Richert & Ryan, 1977a,b), along with a range of Tos-Arg-OMe concentrations (1–10 mM), failed to reverse the inhibitory action of the Tos-Arg-OMe (results not shown); however, washing of rat ovarian 2000g membrane preparations exposed to 5 mM-Tos-Arg-OMe restored human choriogonadotropin stimulation (Table 7). Nearly total inhibition of human choriogonadotropin-stimulated adenylate cyclase by this concentration of Tos-Arg-OMe (2 mM, 1:2.5 dilution in assay) required a 30 min incubation at 30°C, as would be expected from Table 5. In addition, it was necessary to resuspend the membranes in buffer at room temperature in order to reverse the effect of the added Tos-Arg-OMe.

Table 7. *Removal of Tos-Arg-OMe from 2000 g membrane pellet by washing*

Rat ovarian membranes were brought to 5 mM-Tos-Arg-OMe by the addition of an equal volume of 10 mM Tos-Arg-OMe in Tris/HCl buffer; control membranes received an equal volume of Tris/HCl buffer. The preparations were incubated for 30 min at 30°C, and a portion was removed from both and kept on ice. The remaining membranes were pelleted by centrifugation (15 min at 5000g), the supernatants carefully removed and discarded, and the pellets resuspended in 40 mM-Tris/HCl buffer, pH 7.4, at room temperature. After 10 min at room temperature, the resuspended pellets were kept on ice. The original and washed membranes were assayed for adenylate cyclase activity as described in the Materials and Methods section. Final Tos-Arg-OMe concentration in the assay was 2 mM. Results, means of triplicate determinations $\pm$ S.E.M., are expressed as fold stimulation of basal activity.

	Stimulation (fold)			
	Control		+ 5 mM-Tos-Arg-OMe	
	+Human choriogonadotropin	+NaF	+Human choriogonadotropin	+NaF
Original	4.2 $\pm$ 0.5	14.2 $\pm$ 0.8	1.4 $\pm$ 0.1	19.7 $\pm$ 0.6
Washed	2.3 $\pm$ 0.2	25.9 $\pm$ 2.6	2.1 $\pm$ 0.1	31.0 $\pm$ 1.6

Discussion

The original observation that serine proteinases can directly stimulate adenylate cyclase (Hanoune *et al.*, 1977) has been confirmed by a number of laboratories (Richert & Ryan, 1977a; Anderson *et al.*, 1978; Abramowitz & Birnbaumer, 1979). The above, combined with the observation that low-molecular-weight serine proteinase inhibitors block human choriogonadotropin stimulation of rat ovarian adenylate cyclase (Richert & Ryan, 1977b), has led to the suggestion that a proteinase or a cascade of proteinases may be involved in the activation of adenylate cyclase (Richert & Ryan, 1977b; Richert *et al.*, 1978).

It can be argued that small molecules known to interact with proteins should affect a complex system such as the adenylate cyclase system. This argument is especially relevant to the effects of the chloromethyl ketone derivatives and phenylmethanesulphonyl fluoride on adenylate cyclase since these inhibitors are known to interact with free thiol groups (Whitaker & Perez-Villasenor, 1968; Rossman *et al.*, 1974). However, it is unlikely that this is the mechanism by which hormonal stimulation of adenylate cyclase is blocked, since phenylmethanesulphonyl fluoride inhibition of human-choriogonadotropin-stimulated cyclase is not reversed by the addition of dithiothreitol (Fig. 1), and the time course of inhibition by chloromethyl ketone derivatives (Table 5) is much slower than that reported for chloromethyl ketone inhibition of papain (Whitaker & Perez-Villasenor, 1968). This argument (the effect on free thiol groups) should not be applicable to the effects of synthetic substrates such as Tos-Arg-OMe.

Ascoli & Puett (1978a,b) have investigated the role of human choriogonadotropin degradation in

hormonal stimulation of steroidogenesis in a Leydig-tumour cell line and obtained data on the effect of various proteinase inhibitors on 125 I-labelled human choriogonadotropin binding that essentially are in agreement with that reported here—namely, there is no effect on binding. They also examined the ability of some of the inhibitors to block hormonal stimulation of steroidogenesis, but their data are not applicable to the effects reported here on adenylate cyclase, since their end point is one that could be inhibited through the effect of chloromethyl ketone derivatives and phenylmethanesulphonyl fluoride on thiol groups.

The inhibition of adenylate cyclase by Tos-Lys-CH₂Cl has been confirmed by Abramowitz & Birnbaumer (1979); however, they attributed the effect to a combination of the inhibitor and ethanol, which was used as the original solvent. Although this is possible, it has little bearing on the present or previous (Richert & Ryan, 1977b; Richert *et al.*, 1978) studies, since in our hands Tos-Lys-CH₂Cl from the same source was soluble in water. In addition, Abramowitz & Birnbaumer (1979) reported that a higher concentration of Tos-Lys-CH₂Cl (2 mM) was necessary to obtain half-maximal inhibition of human-choriogonadotropin-stimulated adenylate cyclase from rabbit luteal tissue in a 10 min incubation than that reported here for both ovarian and hepatic adenylate cyclase (0.4 mM) in a 20 min incubation (Table 1). This discrepancy is probably a reflection of the method of reporting the effects, since half-maximal inhibitory doses for irreversible inhibitors are very sensitive to the time of the incubations employed to measure them.

It is apparent from the kinetics of inhibition of adenylate cyclase by Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl, Tos-Leu-CH₂Cl and Tos-Arg-OMe that the

inhibitors act at two different sites. The decrease in the initial velocity of hormonally stimulated adenylate cyclase by high concentrations of all the inhibitors examined kinetically (Table 5) suggests that they are acting directly on the active moiety of the adenylate cyclase. This view is confirmed by the action of Tos-Arg-OMe on p[NH]ppG- (Fig. 3) and NaF-stimulated adenylate cyclase (Table 6) where the presence of 10mM-Tos-Arg-OMe caused reversible inhibition of adenylate cyclase, and by the action of Tos-Lys-CH₂Cl on NaF-stimulated adenylate cyclase (Table 6), which required a much higher concentration of the inhibitor than was necessary for inhibition of hormonally stimulated adenylate cyclase.

Though it may be fortuitous, it should be pointed out that the extent of inhibition of the active ovarian adenylate cyclase moiety by 10mM-Tos-Arg-OMe was approximately the same in all three cases, 47% reduction in V_0 of human choriogonadotropin-stimulated adenylate cyclase, 53% reduction for NaF-stimulated adenylate cyclase and 40–60% reduction for p[NH]ppG-stimulated adenylate cyclase.

The irreversible effect of the three chloromethyl ketone derivatives, Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl and Tos-Leu-CH₂Cl, at low concentrations is not surprising given their known action on serine proteinases (Tobita & Folk, 1967; Whitaker & Perez-Villasenor, 1968; Shaw, 1970; Shaw & Glover, 1970; Barrett, 1977a); however, the apparently irreversible nature of Tos-Arg-OMe inhibition, when it is added to an operating hormonally stimulated adenylate cyclase system, is surprising. It is unlikely that the inhibition depends upon the production of some compound from the added Tos-Arg-OMe, since Tos-Arg, toluenesulphonic acid, toluene and methanol, all of which can be derived from Tos-Arg-OMe, have no or very little effect on adenylate cyclase activity (Table 2; P. J. McIlroy, N. D. Richert & R. J. Ryan, unpublished work). The possibility that the effect can be

attributed to the inability of the molecule to gain rapid access to another site of action is also unlikely given the rapid, direct effect of Tos-Arg-OMe on the active adenylate cyclase moiety and the lower effective concentrations of the chloromethyl ketone inhibitors. The phenomenon is readily explainable if hormonally stimulated adenylate cyclase continually undergoes a cyclic activation=inactivation, similar to that suggested by Cassel & Selinger (1978) and by Johnson *et al.* (1978), in which the inactive to active conversion is inhibited by Tos-Arg-OMe and other proteinase inhibitors. This would trap the adenylate cyclase in an inactive state and make it appear to be irreversibly inhibited. This would be further supported by the observation that Tos-Arg-OMe was acting reversibly, as indicated by the washout experiment (Table 7).

The inhibitory rate constants obtained would then be a measurement of the rate of conversion of the active form of the adenylate cyclase to the inactive form, rather than a direct measurement of the effects of the proteinase inhibitors on the adenylate cyclase moiety that they are affecting. This would account for the fact that all the rate constants generated appear to approach the same value, which is one-half to one-third of the limiting values expected if one plots inhibitor concentrations and the obtained rate constants in a double-reciprocal manner as described by Kitz & Wilson (1962).

Scheme 1 outlines an hypothesis concerning the action of low-molecular-weight proteinase inhibitors on adenylate cyclase. The indirect mechanism is postulated to be through a proteinase or proteinases as previously suggested (Richert & Ryan, 1977b; Richert *et al.*, 1978). This postulate is supported by a variety of information. Low-molecular-weight proteinase inhibitors act to reverse hormonally stimulated adenylate cyclase activity at concentrations that are effective on serine proteinases (Tables 1 and 3; Shaw & Glover, 1970). Phenylmethanesulphonyl fluoride, and probably chloromethyl ketones, do not act through the removal of

Scheme 1. Proposed model for sites of action of proteinase inhibitors on adenylate cyclase

free thiol groups since dithiothreitol fails to reverse inhibition by phenylmethanesulphonyl fluoride. Trypsin, chymotrypsin and other proteinases will activate adenylate cyclase at a site other than the active catalytic moiety (Richert & Ryan, 1977a; Richert *et al.*, 1978). All the compounds examined in Tables 1–4, which have an effect on hormonally stimulated adenylate cyclase, are known proteinase inhibitors (Barrett, 1977a) or can be expected to inhibit proteinases, and all those that are not proteinase inhibitors have no effect. The inability of nucleotides other than p[NH]ppG to reverse the inhibition of adenylate cyclase (Fig. 2) rules out the possibility that the inhibitors act by effectively removing some nucleotide that is necessary for activity.

p[NH]ppG, rather than replacing a nucleotide, acts to stimulate adenylate cyclase activity by a different mechanism (Londos *et al.*, 1974; Cassel & Selinger, 1978). This circumvents the indirect effect of the inhibitors and results in an increase in enzyme activity (Fig. 3a); however, this activity is subject to the direct effect of the inhibitors (Figs. 3a and b).

Although most of the compounds that act at low concentrations are known inhibitors of serine proteinases, others affect different types of proteinases (Tables 1 and 3). Leu-NNAp and its derivatives are substrates for, and thus competitive inhibitors of, arylamidases (Barrett, 1977a), chloroquine is an inhibitor of cathepsin B (Barrett, 1977b), and 1,10-phenanthroline and Leu-Trp are inhibitors of metalloproteinases (Barrett, 1977a). This behaviour reveals a diversity that is difficult to explain. Similar diversity is also seen in the actions of the various amino-acid derivatives examined. There is no obvious specificity in the ability of the derivatives to inhibit hormonally stimulated adenylate cyclase (Tables 1 and 3). In some cases chymotrypsin-specific inhibitors are better inhibitors of adenylate cyclase than trypsin-specific inhibitors (e.g. Tos-Leu-CH₂Cl versus Tos-Lys-CH₂Cl, Table 1), whereas in other cases the reverse is true (e.g. *N*-Ac-Arg-OMe versus *N*-Ac-Leu-OMe, Table 3). Unblocked *N*-acetylated amino acids appear to be better inhibitors than their methyl ester counterparts (Table 3). This diversity may be a manifestation of the involvement of more than one enzyme in the mechanism of hormonal stimulation (Richert & Ryan, 1977b; Richert *et al.*, 1978), or alternatively, it may be a reflection of the inhibition of a highly specific proteinase whose active site binds a three- or four-amino-acid sequence.

The proposed model is equally applicable to other adenylate cyclase systems. The ability of the various inhibitors to inhibit glucagon stimulation of rat liver adenylate cyclase (Table 1) supports this view, and shows that the effect of proteinase inhibitors on adenylate cyclase is not confined to the ovary.

Previous results also indicated that the inhibitors blocked catecholamine and cholera-toxin activation in ovarian tissue (Richert & Ryan, 1977b; Richert *et al.*, 1978).

This work was supported by funds from the National Institute of Child Health and Human Development (HD 9140) and the Mayo Foundation. We thank Dr. A. R. LaBarbera for many helpful discussions and Mr. K. D. Peters and Mrs. E. R. Bergert for excellent technical assistance.

References

- Abramowitz, J. & Birnbaumer, L. (1979) *Biol. Reprod.* **21**, 213–217
- Anderson, W. B., Jaworski, C. J. & Vlahakis, G. (1978) *J. Biol. Chem.* **253**, 2921–2926
- Ascoli, M. & Puett, D. (1978a) *J. Biol. Chem.* **253**, 7832–7838
- Ascoli, M. & Puett, D. (1978b) *J. Biol. Chem.* **253**, 7839–7843
- Barrett, A. J. (1977a) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 1–55, Elsevier/North-Holland, Amsterdam
- Barrett, A. J. (1977b) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 209–248, Elsevier/North-Holland, Amsterdam
- Birnbaumer, L., Yang, P.-C., Hunzicker-Dunn, M., Bockaert, J. & Duran, J. M. (1976) *Endocrinology* **99**, 163–184
- Cassel, P. & Selinger, F. (1978) *Proc. Nat. Acad. Sci. U.S.A.* **75**, 4155–4159
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Guiraud-Simplot, A. & Golobert, L. (1977) *Experientia* **33**, 899–901
- Hanoune, J., Stengel, D., Lacombe, M.-L., Feldman, G. & Coudrier, E. (1977) *J. Biol. Chem.* **252**, 2039–2045
- Johnson, G. L., Kaslow, H. R. & Bourne, H. R. (1978) *Proc. Nat. Acad. Sci. U.S.A.* **75**, 3113–3117
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Lad, P. M., Welton, A. F. & Rodbell, M. (1977) *J. Biol. Chem.* **252**, 5942–5946
- Laidler, K. J. & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, Clarendon Press, Oxford
- Lee, C. Y. (1976) *Endocrinology* **99**, 42–48
- Lee, C. Y. & Ryan, R. J. (1973) *Biochemistry* **12**, 4609–4615
- Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. & Randall, M. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3087–3090
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McIlroy, P. J. & Ryan, R. J. (1979) *Abstr. Annu. Meet. Endocrine Soc.* 61st abstr. 847
- Richert, N. D. & Ryan, R. J. (1977a) *Proc. Nat. Acad. Sci. U.S.A.* **74**, 4857–4861
- Richert, N. D. & Ryan, R. J. (1977b) *Biochem. Biophys. Res. Commun.* **78**, 799–805

- Richert, N. D., Bramley, T. A. & Ryan, R. J. (1978) in *Novel Aspects of Reproductive Physiology* (Spilman, C. H. & Wilks, J. W., eds.), pp. 81–106, S. P. Medical & Scientific Books, New York
- Rossmann, T., Norris, C. & Troll, W. (1974) *J. Biol. Chem.* **249**, 3412–3417
- Ryan, W., Short, N. A. & Curtis, G. L. (1975) *Proc. Soc. Exp. Biol. Med.* **150**, 699–702
- Shaw, E. (1970) *Physiol. Rev.* **50**, 244–296
- Shaw, E. & Glover, G. (1970) *Arch. Biochem. Biophys.* **139**, 298–305
- Tobita, T. & Folk, J. E. (1967) *Biochim. Biophys. Acta* **147**, 15–25
- Whitaker, J. R. & Perez-Villasenor, J. (1968) *Arch. Biochem. Biophys.* **124**, 70–78