

BIOCHEMICAL AND MECHANICAL EFFECTS
OF ADRENERGIC AND HISTAMINERGIC DRUGS

by

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ABSTRACT

A time-response and dose-response study of the effects of norepinephrine and phenylephrine revealed that both agonists caused increases in cyclic AMP, cardiac contractility and phosphorylase a in the isolated perfused guinea pig hearts. Norepinephrine caused a nearly five fold increase in cyclic AMP, whereas phenylephrine produced only a two-three fold increase in the nucleotide. Phenylephrine is less potent and less effective in elevating all the three parameters as compared to norepinephrine.

Histamine and its analogs, TD and betazole increased cardiac contractility, phosphorylase a and levels of cyclic AMP in the isolated perfused guinea pig heart. The order of potency for the three compounds was histamine>TD>betazole. Cyclic AMP was found to increase prior to the increase in contractility or phosphorylase a. In the present study, the new H₂-receptor blocking agent burimamide, was found to be a specific, competitive blocking agent of both the mechanical and biochemical effects of histamine and its analogs on the heart. Burimamide did not affect the norepinephrine-induced increase in contractility, phosphorylase a or cyclic AMP. Promethazine did interact with cardiac histamine receptors, but the interaction was either non-competitive or competitive non-equilibrium in nature.

Histamine, 4-methylhistamine, TD and betazole all stimulated cardiac adenylate cyclase in doses ranging from 10^{-7} to 10^{-3} M of the agonists. The order of potency of the compounds for stimulating the enzyme was histamine>4-methylhistamine>TD>betazole. Stimulation by the agonists was blocked in an apparently competitive manner, by burimamide, since burimamide (1×10^{-6} - 1×10^{-5} M) produced progressive shifts in the dose-response curves of the

agonists to the right.

Histamine, 4-methylhistamine, TD and betazole, in that order increased rat gastric adenylate cyclase activity in doses ranging from 10^{-7} to 10^{-3} M of the agonists. Burimamide an H_2 -receptor blocking agent, in concentrations of $1-5 \times 10^{-6}$ M antagonized the drug-induced enzyme stimulation. The maximum increase in the enzyme activity was approximately three fold and was competitively blocked by burimamide.

The relative order of these agonists in stimulating cardiac or gastric adenylate cyclase was similar to that found when the effect of these agents on cardiac cyclic AMP, contractility and phosphorylase a was measured.

Histamine and histamine analogs relaxed the rat uterus in a dose-dependent manner. The rank order was histamine > 4-methylhistamine > TD and betazole. However histamine did not produce the activation of the adenylate cyclase prepared from rat uterus prepared from rat uterus.

Histamine (10^{-5} M), produced the maximum response in the guinea pig ileum, which was blocked by tripeleennamine and diphenhydramine (H_1 -receptor antagonists), but not by burimamide (H_2 -receptor antagonist). Histamine did not produce any changes in cyclic AMP levels in the isotonically contracting guinea pig ileum.

The data provide some evidence that H_2 -receptors have similar properties in at least two tissues, gastric mucosa and heart. The data also provide further evidence for the association of the H_2 -receptor with adenylate cyclase and dissociation of adenylate cyclase from H_1 -receptors.

The cardiac effects of the catecholamines, and histamine may be mediated through cyclic AMP. The effects of the amines are potentiated or inhibited by the drugs which affect the enzyme phosphodiesterase. Theophylline injection (1 mg) into the perfused guinea pig heart resulted in an increase

in contractility of about 20% over the control. Theophylline also potentiated the inotropic and phosphorylase a producing effects of norepinephrine and histamine but did not produce any changes in cardiac cyclic AMP.

Imidazole, a phosphodiesterase stimulator, also caused an increase in cardiac contractility. Imidazole perfusion (40 mM) decreased or abolished the positive inotropic effect of norepinephrine and histamine, and produced a parallel fall in the amine-induced increase in cardiac cyclic AMP. However imidazole perfusion did not affect the phosphorylase activating effect of either amine. The effect of amines on phosphorylase activation in the presence of imidazole can not be explained on the basis of cyclic AMP. Both, theophylline and imidazole produce effects on the hearts which are apparently unrelated to their known effects on cyclic AMP phosphodiesterase. It is known that the methylxanthines release calcium from intracellular storage sites and that imidazole increases the influx of calcium. It is evident from our data that both theophylline and imidazole produced positive inotropic responses which can be interpreted in terms of the increased calcium influx.

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
AMP	adenosine monophosphate
cyclic AMP	adenosine 3',5'-cyclic monophosphate
db. cyclic AMP	dibutyryl adenosine 3',5'-cyclic monophosphate
GTP	guanosine 5'-triphosphate
cyclic GMP	guanosine 3',5'-cyclic monophosphate
G-1-P	glucose-1-phosphate
Pi	inorganic phosphate
TD	3-(β -aminoethyl)1,2,4 triazole
Tris	tri(hydroxymethyl)aminomethane
ppo	2,5-diphenylonazole
popop	1,4-bis[2-(5-phenylonazolyl)]-benzene

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Dedication

To Asha and Subodh

Review of Literature

A. Relationship between hormone and cyclic AMP

Complex physiological and metabolic processes in living organisms are partially controlled by hormones. The question as to how hormones act was posed by Sutherland, Rall and their colleagues. The study of glucagon or epinephrine-induced mobilization of liver glycogen led to a possible partial answer to this question. The addition of these hormones to liver slices caused accumulation of a heat stable, dialysable adenosine nucleotide, which eventually was identified as adenosine 3',5' cyclic monophosphate, commonly referred to as cyclic AMP (Sutherland et al. 1962). Extensive research revealed that cyclic AMP was synthesized following the action of certain hormones on adenylate cyclase, a plasma membrane bound enzyme, distributed in many mammalian tissues (Sutherland et al. 1962). In addition to epinephrine many polypeptide hormones increase the concentration of cyclic AMP or stimulate adenylate cyclase activity in target tissues Fig.(1). These findings led to the second messenger hypothesis of hormone action (Sutherland, Øye and Butcher 1965).

The hormone or first messenger binds to the specific receptor in the plasma membrane and activates adenylate cyclase, which is located in close proximity to the specific hormone receptor. Adenylate cyclase stimulation by the appropriate hormone results in an increased conversion of (Mg^{++}, ATP^{++}) to cyclic AMP, which is referred to as a second messenger. The nucleotide influences specific protein kinase to donate phosphate moieties to those effector proteins which ultimately carry out the specific cellular transactions (Walsh et al. 1968; Garren et al. 1971; Bitensky et al. 1973; Butcher and Sutherland 1971). The activation of adenylate

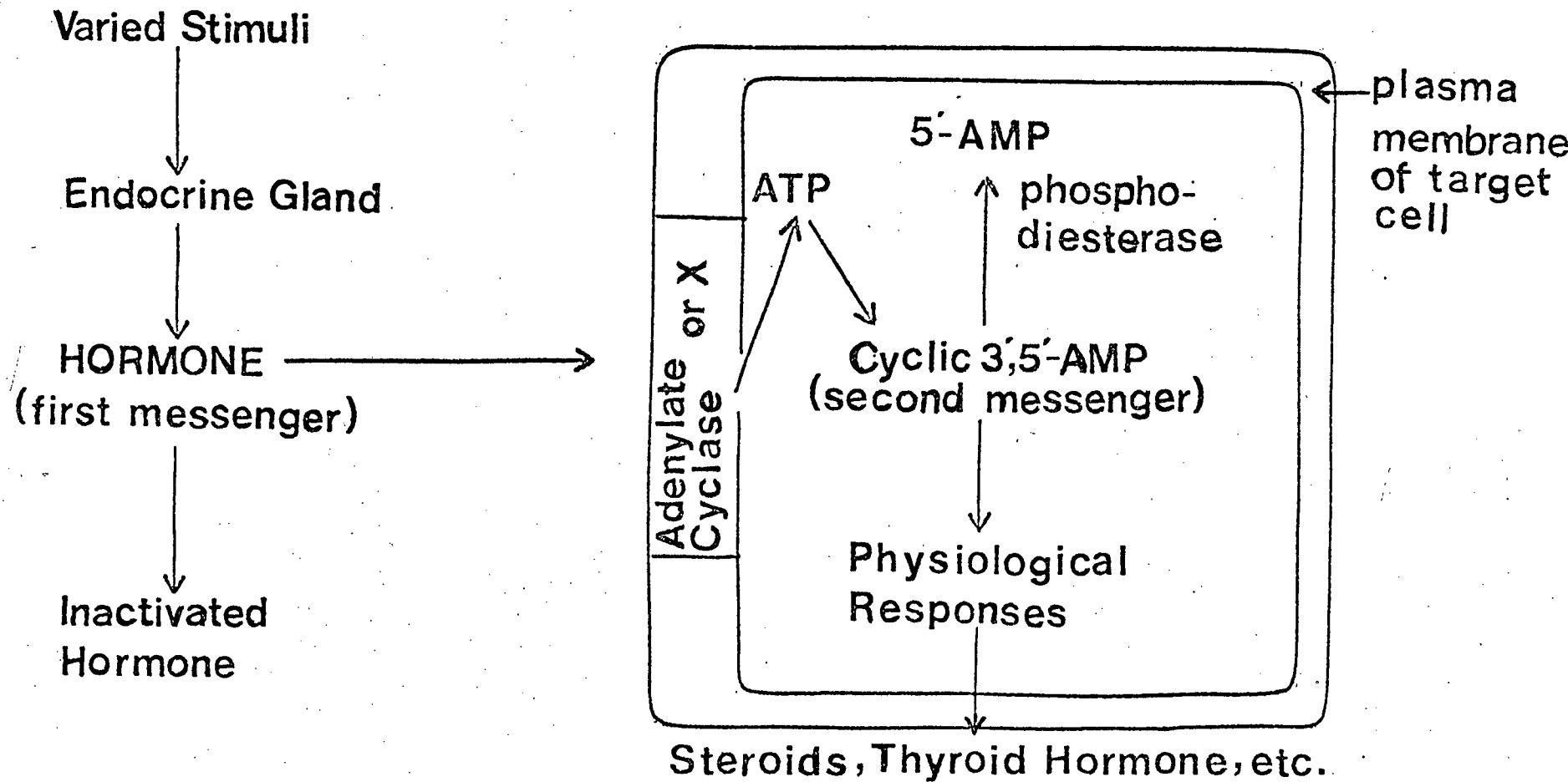


Fig.1. Schematic representation of the second messenger concept

cyclase is specific in nature (Lefkowitz 1973). Adrenocorticotrophic hormone will activate adrenal adenylate cyclase; parathormone increases adenylate cyclase in renal cortex and vasopressin in renal medulla (Chase 1968). Glucagon, catecholamines and histamine all activate cardiac adenylate cyclase (Murad and Vaughan 1969; Levey and Epstein 1969; Mayer et al. 1970; Sutherland et al. 1968; Pösch and Kukovetz 1967; Klein and Levey 1971; McNeill and Muschek 1972).

Glucagon and epinephrine stimulate liver adenylate cyclase (Birnbaumer and Rodell 1969). In liver, two entirely separate adenylate cyclase systems exist for glucagon and epinephrine. The enzyme is also stimulated non-specifically by fluoride. Fluoride stimulation occurs in enzyme obtained from any tissue (Sutherland et al. 1962) and the fluoride activated enzyme cannot be further stimulated by hormones specific to that tissue (Sutherland et al. 1962; Birnbaumer et al. 1969).

The intracellular concentration of cyclic AMP is determined by a balance in activities of two enzymes, adenylate cyclase and phosphodiesterase, the enzyme responsible for the metabolism of cyclic AMP to 5' AMP. The activity of both these enzymes can be affected by drugs.

In summary, the demonstration of the presence of adenylate cyclase in most mammalian tissues and the fact that a given hormone can increase enzyme activity provides suggestive evidence that the adenylate cyclase-cyclic AMP system may serve as the mediator for the action of that particular hormone on its target tissue. However, before a reasonable degree of credibility can be attributed to such an hypothesis four criteria, as defined by Sutherland and his coworkers (1962), should be fulfilled:

1. Demonstration of a response to the hormone in a washed broken cell preparation.

2. An appropriate increase in cyclic AMP in intact cells in response to hormone stimulation.
3. Drugs affecting phosphodiesterase should potentiate or inhibit the hormone action.
4. Exogenous cyclic AMP or its derivatives should mimic the effect of the hormone.

Tables 1, 2, summarises the possible involvement of cyclic AMP as a mediator of the actions of various hormones.

TABLE 1

Possible Involvement of Cyclic AMP in Hormone Actions
on Various Tissues.

<u>Tissue</u>	<u>Hormone</u>
Liver	Glucagon
Adrenohypophysis	Hypothalamic releasing hormone
Renal medulla	Vasopressin
Adrenal Cortex	Adrenocorticotrophic hormone
Interstitial Cells	Luteinizing hormone
Semeniferous tubules	Follicle-Stimulating hormone
Melanophores	Melanocyte-Stimulating hormone
Thyroid	Thyroid-Stimulating hormone
Bone and renal cortex	Parathyroid hormone
Brain	Histamine, Serotonin

Catecholamines, stimulate many target tissues (Robison, G.A. 1972).

TABLE 2

Possible Involvement of Cyclic AMP in Various
Metabolic Processes.

Protein kinase activation
Phosphorylase activation
Stimulation of glyconeogenesis
Stimulation of ketogenesis
Stimulation of lipolysis
Stimulation of steroidogenesis
Stimulation of exocytosis
Inhibition of glycogen synthetase activity
Inhibition of lipogenesis
Inhibition of cell growth
Inhibition of platelet aggregation
Inhibition of cerebellar purkinje cell firing
Increased permeability to water and electrolytes
Increased force of cardiac contractility

(Robison, G.A. 1972).

Of the metabolic effects in which cyclic AMP is supposed to be involved, only a few are understood. The most studied is the activation of the enzyme glycogen phosphorylase in skeletal muscle (Krebs et al. 1966; Walsh et al. 1968). Cyclic AMP increases the activity of a protein kinase (Walsh et al. 1968) which catalyses the activation of phosphorylase b kinase. Phosphorylase b kinase in turn catalyzes the phosphorylation of inactive phosphorylase b to form active phosphorylase a. Phosphorylase a in turn converts glycogen to G-1-P (Fig. 2).

Cyclic AMP-sensitive protein kinases seem to be at least as widely distributed as cyclic AMP itself (Kuo and Greengard 1969) and many proteins other than phosphorylase and glycogen synthetase can serve as substrates for them. It seems possible, therefore, that many if not most of the physiologically important effects of cyclic AMP in mammalian cells may be brought about by the activation of a protein kinase.

In consideration of the vast amount of information involving cyclic AMP as a second messenger, this introduction will be directed solely to the postulated role of cyclic AMP in the effects of adrenergic and histaminergic drugs.

B. Adrenergic cardiac effects

The cardiac effects of several hormones have been postulated to involve an interaction with cardiac adenylate cyclase. In particular catecholamines have been the subject of intensive investigation (Robison, Butcher, Øye, Morgan and Sutherland 1965; Williamson 1966; Sutherland et al. 1966; Drummond et al. 1966; Mayer 1972). Catecholamine effects can be classified into metabolic and mechanical effects. The most studied metabolic effect is the activation of phosphorylase. The transformation of the enzyme

CYCLIC AMP

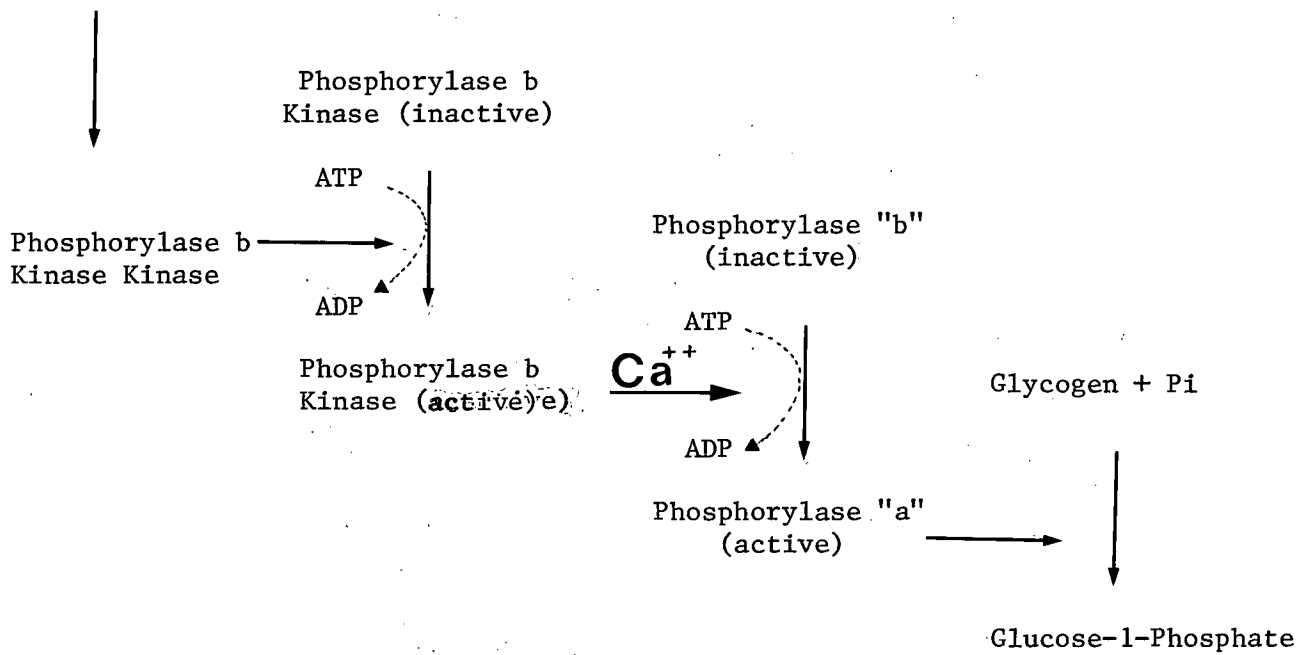


FIG. 2. Enzymes involved in the control of myocardial glycogenolysis

phosphorylase b to the phosphorylated form phosphorylase a, has been used as an index of an increase in cyclic AMP in myocardial cells.

Various workers have studied the relative potencies of adrenergic drugs on a wide range of species. Lands and Howard (1952), reported that epinephrine was more effective than norepinephrine in increasing the amplitude and rate of contraction in the perfused frog heart and tortoise atria. Epinephrine was less effective than norepinephrine on isolated rabbit atria and perfused rabbit heart, but isoproterenol was much more effective than epinephrine or norepinephrine in producing increases in rate and amplitude of contraction in all the above preparations (Nickerson and Mullenberg, 1967; Robison et al. 1970). Catecholamine-induced increases in cardiac contractility have been studied by electrophysiologists in terms of the electrical events at the cell membrane (Edman 1965). Catecholamines increased the fluxes of Na^+ and K^+ across the cell membrane (Glitsch et al. 1965). An interesting approach to explain the mode of inotropic action of catecholamines, concerns the catecholamine-induced changes in the metabolism of cardiac muscle glycogen. This mechanism explains how the energy is made available for increased cardiac contraction. Catecholamine effects are exerted upon a complex system, referred to as the cyclic AMP-phosphorylase system, which is the target for the action of several drugs (Hess and Haugaard 1958). Hess and Haugaard (1959) reported that epinephrine caused an increase in phosphorylase activity and a positive inotropic effect in an isolated rat heart preparation. Kukovetz, Hess, Shanfeld and Haugaard (1959) reported that the inotropic effect and phosphorylase activation were closely associated. Both of these actions were related in a dose-dependent manner. Kukovetz et al. (1959) have presented a linear relationship between the positive inotropic effect and phosphorylase activity. Mayer and Moran

(1960) confirmed the above findings in the open-thorax dog heart preparation and reported that isoproterenol was approximately ten times as potent as norepinephrine or epinephrine.

Hess et al. (1962) found no dissociation between the inotropic responses and phosphorylase activation in the rat heart, even with small doses of epinephrine (0.005-1.0 μ g). However Øye et al. (1964) claimed that the inotropic response to the catecholamines could be dissociated from phosphorylase activation by the use of very small doses of epinephrine and norepinephrine. Mayer et al. (1963) supported the findings of Øye et al. (1964) when they reported that in the open-thorax dog heart preparation norepinephrine produced significant inotropic effects with no measurable changes in phosphorylase a activity. Shanfeld, Frazer and Hess (1968; 1969) were able to dissociate the contractility of the heart induced by norepinephrine from the effects of the drug on myocardial cyclic AMP content. They were successful in blocking the phosphorylase activation of norepinephrine by N-isopropylmethoxamine ((IMA), without the inotropic responses being significantly depressed. These findings were questioned. Wastila et al. (1972) suggested that the results obtained by Shanfeld et al. (1969) could be an artifact introduced by their readjustment of the diastolic tension after the administration of IMA to the perfused isolated rat heart. Moreover the concentration used (15 μ g/ml) in their study was probably cardiodepressant. In a similar study, Robison et al. (1965) using IMA in the isolated perfused rat heart, reported that ~~thene~~epinephrine-induced rise in cyclic AMP or contractility was not blocked by IMA. Murad et al. (1962) correlated adenylate cyclase activation by drugs to their positive inotropic effect. The relative potencies of isoproterenol¹, norepinephrine and epinephrine in activating adenylate cyclase were similar to those found

when phosphorylase activation was obtained (Murad et al. 1962). Epinephrine increased the levels of cyclic AMP in the rat heart and the increase preceded the other adrenergic effects such as the inotropic, chronotropic and phosphorylase activation effects (Øye et al. 1964; Robison 1965).

Williamson (1966) studied the kinetic changes following the injection of 1 µg of epinephrine into the rat heart in a Langendorff preparation. Within 2 sec. of the epinephrine injection cyclic AMP and contractile force both increased two fold, but phosphorylase levels did not change. Phosphorylase a levels increased and reached a broader peak at 20-30 sec. Cyclic AMP levels continued to rise, reached a sharp peak at 10 sec. and abruptly decreased to control values within 20 sec. Both contractility and cyclic AMP reached their peak at about the same time. Williamson's findings suggested a causal relationship between the three biochemical and mechanical parameters.

It has been shown that stimulation of beta-adrenergic receptors leads to the activation of adenylate cyclase and an increase in the tissue levels of cyclic AMP (Rall and Sutherland 1961; Mayer et al. 1963; Robison et al. 1971; Greengard and Robison 1972; Lefkowitz, Roth and Pastan 1971; Lefkowitz, Sharp and Haber 1973; Lefkowitz and Levey 1972). It has been suggested that adenylate cyclase is in fact, the beta-adrenergic receptor (Robison et al. 1966; Mayer 1970; Robison et al. 1971). Evidence has been presented by Lefkowitz and Haber (1971) and Lefkowitz and Levey (1972) that beta-receptors are separate and distinct from adenylate cyclase. Levey (1971), Levey and Klein (1972), Lefkowitz (1971, 1973) separated the enzyme adenylate cyclase and beta-adrenergic receptors. A solubilized enzyme preparation was hormonally unresponsive and responses could be restored upon the addition of phospholipids but the binding of [³H] epinephrine to the receptor did not

require phospholipid. On the basis of their data, it appears that the initial interaction of norepinephrine is with cardiac beta-receptors, which are coupled to adenylate cyclase by a phospholipid linkage.

There is a specific phospholipid linkage for each respective hormone e.g. phosphatidylserine for glucagon and histamine and monophosphatidylinositol for norepinephrine (Levey 1971).

Positive support for the third criterion, i.e. potentiation of the amine effects by theophylline, a phosphodiesterase inhibitor was given by Rall and West (1963) who noted that the inotropic effect of norepinephrine was greatly enhanced in the presence of theophylline. Antagonism of the positive inotropic effect, by imidazole, a potent stimulator of phosphodiesterase (Butcher and Sutherland 1962), provided further evidence for cyclic AMP mediation of the catecholamine action (Kukovetz and Poch 1967).

The fourth criterion is that cyclic AMP or its derivative should have the ability to produce the end organ response directly. Use of the dibutyryl derivative of cyclic AMP led to the fulfilment of the fourth criterion for the myocardium (Epstein 1970; Skelton et al. 1969; Skelton et al. 1970). Drummond and Hemmings (1972) reported that perfusion of 0.2 mM db. cyclic AMP into the isolated heart caused a positive inotropic effect within about 2 - 4 min. The maximum effect occurred at concentrations of 2 - 3 mM of db. cyclic AMP. Skelton, Levey and Epstein (1970) reported that db. cyclic AMP exhibited a positive inotropic effect on isolated cat papillary muscle driven electrically. A study by Krause et al. (1970) demonstrated that db. cyclic AMP produced both positive inotropic and chronotropic responses in cultures of spontaneously beating heart cells.

Thus all four of Sutherland's criteria have been satisfied implicating

cyclic AMP as a mediator of the positive inotropic action of the catecholamines. One of the suggestive sites for cyclic AMP action is the sarcoplasmic reticulum. Myocardial sarcoplasmic reticulum exhibits adenylate cyclase activity (Katz et al. 1970). Entman et al. (1969) have suggested that cyclic AMP increases calcium accumulation by the sarcoplasmic reticulum and that this effect contributes to the positive inotropic effect of the catecholamines. However the cyclic AMP-induced stimulation of calcium accumulation has not been confirmed (Sulakhe and Dhalla 1970). The differences in the data of Entman et al. (1969) and Sulakhe and Dhalla (1970) could be due to the difference in the preparation of sarcoplasmic reticulum. Preparations of sarcoplasmic reticulum are extremely difficult to purify and the possibility of contamination with plasma membrane fragments, a likely source of the observed enzyme activity, can not be excluded.

Electrophysiological and tracer studies have demonstrated that catecholamines increase the transmembrane flux of calcium ions into the myocardial cells (Engstfeld et al. 1961; Renter 1967; Vassort et al. 1969; Carmeliet and Vereecke 1969; Pappano 1970).

It is generally accepted that catecholamines also increase cyclic AMP levels in cardiac tissue (Sutherland et al. 1968). This increase in the intracellular levels of cyclic AMP and the membrane calcium influx appear to be concurrent effects of catecholamines on the myocardial cells.

Rasmussen, Goodman and Tenenhouse (1972) have cited the importance of calcium along with cyclic AMP in adrenergic cardiac effects. Their hypothesis states that stimulation of beta-receptors by catecholamines could simultaneously cause an activation of adenylate cyclase and an alteration of sarcolemmal permeability to Ca^{2+} ; or increased levels of cyclic AMP within the cell could elicit increased sarcolemmal conductance to calcium. In the first type

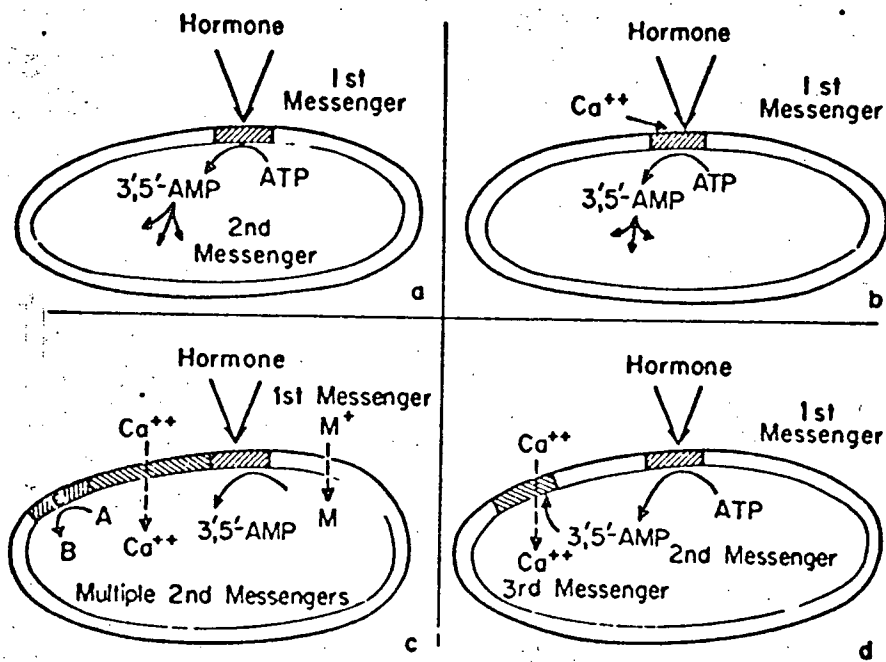


FIG. 3. Possible relationships between hormone-receptor interactions and calcium in effecting an end-organ response: (Rasmussen et al. 1972).

of interaction cyclic AMP and Ca^{2+} would both function as second messengers, whereas in the latter case cyclic AMP would be the second messenger and Ca^{2+} would serve as third messenger (Fig. 3). Entman, Levey and Epstein (1969) reported that norepinephrine-induced increases in cyclic AMP levels caused an increase in calcium accumulation. Electrophysiological studies lend support to a direct effect of cyclic AMP on calcium transport. Coraboeu (1969) showed that cyclic AMP increased the height of the plateau of the action potential in frog atrial fibres, which is indicative of an increase in calcium inward current through the 'slow $\text{Na}^+ - \text{Ca}^{++}$ channels' of the plasma membrane. In voltage clamp studies in calf purkinje fibres, Tsein (1972) concluded that db. cyclic AMP increased the inward current of calcium. Meinertz et al. (1973) showed that in electrically driven left auricles from rat heart, suspended in Tyrode solution with reduced calcium concentration, db. cyclic AMP as well as epinephrine not only increased the force of contraction but also caused an increase in calcium uptake. A recent study by Watanabe and Besch (1974), suggested that cyclic AMP can activate slow Ca^{2+} channels.

In cardiac glycogenolysis, cyclic AMP operates by regulating phosphorylase b kinase, but calcium probably plays a role in stimulating the phosphoprotein product of the cyclic AMP dependent protein kinase; i.e. the activated form of phosphorylase b kinase. In cardiac glycogenolysis, both calcium and cyclic AMP appear to act sequentially. Disorders of cellular calcium metabolism, such as occur in adrenalectomized animals (Miller, Exton and Park 1971) led to a partial inhibition of the physiological response without changes in the initial rise in cellular cyclic AMP concentration.

In summary, the bulk of the evidence supports the second messenger

hypothesis proposed by Sutherland et al. (1968), that cyclic AMP may be involved in the effects of beta adrenergic amines on the contractile processes of the heart.

Recent reports however [Benfey and Carolin (1971), Benfey (1971)] suggest that phenylephrine does not activate the cardiac enzyme adenylate cyclase, and hence does not produce any changes in the intracellular levels of cyclic AMP. Benfey (1971) also failed to observe any changes in cardiac glycogenolysis, but he reported that phenylephrine does increase cardiac force of contraction. The lack of relationship between cyclic AMP (ventricles) and inotropic effects (atrium) may reflect differences between ventricular and atrial (muscle) rabbit heart rather than prove a dissociation between cyclic AMP and the inotropic effects of sympathomimetic amines. Moreover Benfey (1971) did not measure cyclic AMP in a beating heart in which physiological parameters could be simultaneously monitored. A study by McNeill and Davis (1972) suggests that phenylephrine does activate cardiac phosphorylase as well as producing a positive inotropic effect.

Benfey (1971) claims that his results refute the theory that cyclic AMP acts as a second messenger in the heart and mediates the inotropic effect of the sympathomimetic amines. Since these data do not support the second messenger theory, it was decided to reinvestigate the problem. In the present investigation, time-response and dose-response studies were carried out and the cardiac effects of phenylephrine were compared with those obtained with norepinephrine.

C. Histaminergic effects:

I Cardiac effects of histamine:

Dale and Laidlaw in 1910, reported that histamine caused an increase

in cardiac contractility in the isolated heart of the cat and rabbit. Later on Went and Lissak (1935) and Tiffeneau (1941) reported similar effects of histamine on guinea pig and frog heart. These effects of histamine were reported to be very specific (Mannaioni 1960). In an isolated guinea pig atrial preparation, dichloisoprenaline, a beta-blocker did not antagonize the cardiac histamine effects and in reserpine-treated animals histamine also produced cardiac effects. Trendelenburg (1960) reported that the classical antihistamines, mepyramine and tripeleennamine did not antagonize cardiac histamine effects. But Hughes and Coret (1972) could block cardiac histamine effects by promethazine. Other actions of histamine such as contraction of guinea pig ileum and bronchi (Arunlakshana and Schild 1954) are blocked by the antihistaminic drugs. Like the cardiac histamine effects, histamine effects on gastric acid secretion and relaxation of the rat uterus are not blocked by mepyramine and tripeleennamine (Ash and Schild 1966). From these observations Ash and Schild (1966) classified histamine effects, which are blocked by classical antihistamines, as being due to stimulation of " H_1 " type histamine receptors. The search was continued in order to discover a specific histamine antagonist which can antagonize the effects of histamine on the heart and gastric acid secretion, which apparently are controlled by a separate or H_2 -type of receptor.

Black et al. (1972) in their classical publication reported that burimamide (N-methyl-N'-(4-(4,5)-imidazolyl)butyl thiourea) is a compound which specifically antagonizes the cardiac histamine effects. The cardiac histamine receptors are reported to be different in different species (El-Ackad et al. 1974). Histamine H_2 type of receptors are reported to be present in guinea pig, cat and human heart (Klein and Levey 1971). Rat heart however does not possess histamine receptors (Green and Erickson 1967).

HISTAMINERGIC RECEPTORS (H_1 and H_2) and COMPETITIVE ANTAGONISTS

	H_1 RECEPTORS			H_2 RECEPTORS		
	Guinea-pig ileum	Rat stomach	Man blood pressure	Rat uterus	Guinea-pig atrium	Rat, man gastric secretion
Mepyramine	++	++	+	o	o	o
Burimamide	o	o	+	++	++	+++
Metiamide	o	o	+	++	++	++

FIG. 4. Responses of H_1 and H_2 receptors and their selective inhibition by antagonists (according to Black et al. 1972).

El-Ackad et al. (1974) reported that tripeleennamine and mepyramine (H_1 -antagonists) abolished the histamine-induced increase in heart rate and contractility in avian heart, whereas burimamide (H_2 -antagonist) failed to alter the chronotropic response produced by histamine. Histamine effects were unaltered by reserpine treatment in avian heart. Histamine thus stimulates avian heart by its action on ' H_1 ' type receptors.

Pösch and Kukovetz (1967) suggested the receptors for cardiac histamine effects in guinea pig heart are associated with adenylate cyclase. Dean (1968) postulated that the cardiac effects of histamine are mediated by the adenylate cyclase-cyclic AMP system. Klein and Levey (1971) indicated that histamine has the capacity to activate adenylate cyclase in the particulate fractions of guinea pig, cat and human heart homogenates. Klein and Levey (1971) found that histamine in equimolar concentrations was about as effective as norepinephrine. McNeill and Muschek (1972), reported that histamine induced activation of adenylate cyclase was not blocked by the antihistamines diphenhydramine and tripeleennamine. Theophylline potentiated the cardiac biochemical and mechanical effects of histamine. McNeill and Muschek's (1972) data provided good evidence that histamine fulfils the criteria as previously discussed for a cyclic AMP mediated positive inotropic effect on the heart although histamine seems to act on adenylate cyclase at a site which is different from the adrenergic beta-receptor. In the present study, the above working hypothesis was investigated. Time-response and Dose-response effects of histamine and its analogs, 3-(β -aminoethyl)1,2,4 triazole (TD), and betazole, on cardiac contractility, phosphorylase activation and cyclic AMP were studied. The interaction of burimamide with histamine and its analogs on the above parameters was also investigated. To further characterize the cardiac histamine receptor, the interaction of

histamine on cardiac adenylate cyclase was studied. The nature of blockade by burimamide of the effects of histamine, 4-methylhistamine (a specific H_2 agonist), TD and betazole was also investigated.

Histamine also stimulates the secretion of the acid by the stomach (Ivy et al. 1925; Ash et al., 1966). This effect is also not blocked by classical antihistamines. Therefore it is probably an H_2 type of receptor. The following section is an attempt to discuss the work supporting the concept of H_2 receptors in the stomach.

II Gastric effects of histamine:

Evidence that histamine acts as a chemical mediator for gastric HCl secretion was provided by Ivey and Farrel (1925). They reported that histamine acts directly upon the autotransplanted fundic pouch. Davies (1948) showed that histamine stimulated isolated rat gastric mucosa in vitro. Code (1947) reported a relationship between the output of histamine in the gastric juice and the volume of HCl secretion, when the gastric secretion is stimulated by a meal. Alonso and Harris (1965), reported the activation of gastric acid secretion from gastric mucosa due to gastrin and methylxanthines. Perfusion of rat gastric mucosa with cyclic AMP caused an acid secretion (Shaw and Ramwell 1968). Bersinbaev et al. (1971) observed that pentagastrin or histamine treatment produced an increase in adenylate cyclase activity in the rat gastric mucosa. The studies of Perrier and Laster (1969, 1970), showed that histamine and the histamine analog, betazole stimulated parietal mucosal adenylate cyclase. Karppanen and Westermann (1973) reported that histamine caused an increase in gastric cyclic AMP in the guinea pig. Levine and Washington (1973) suggested that since stimulation of acid secretion was accompanied by an increased production of cyclic AMP in gastric juice, it

appeared likely that cyclic AMP plays a mediating role in the human gastric secretory response to histamine and betazole. Bieck et al. (1973), using denervated pouches of the stomach fundus (Heidenhain pouch dogs) reported that histamine produced a dose dependent elevation of cyclic AMP and hydrochloric acid secretion. He further reported a causal relationship between cyclic AMP elevation and the acid output. Karppanen and Westermann (1973) reported that H_1 receptor blocking agent, diphenhydramine even in a concentration as high as $3.3 \times 10^{-4} M$ only slightly inhibited the histamine stimulated production of cyclic AMP. Diphenhydramine did not inhibit histamine secretion of gastric acid. The histamine-induced gastric acid secretion in rats (Black et al. 1972) and in man (Wyllie et al. 1972) is suggested to be due to H_2 receptor stimulation. Burimamide competitively antagonized the histamine stimulated acid secretion (Wyllie et al. 1972). Another orally active histamine H_2 receptor antagonist metiamide (Black et al. 1973; Dousa and Code, 1974) inhibited gastric secretion apparently by blocking the stimulation of cyclic AMP formation by histamine and its methyl derivative in the gastric mucosa of guinea pig.

The present study was undertaken to investigate the effects of Histamine, 4-methylhistamine TD and betazole on rat gastric adenylate cyclase and to ascertain the interaction of these drugs with burimamide.

Histamine relaxes the rat uterus and the histamine-induced relaxation is not blocked by classical antihistamines (Ash and Schild 1966). Black et al. (1972) suggested the presence of H_2 type of receptors in rat uterus. In the next section the nature of the histamine-receptor in the rat uterus is discussed.

III Histamine-rat uterus interaction:

The finding that histamine caused relaxation of the rat uterus has been reported by Dale and Dudley (1921); Huebner et al. (1949) and Craver et al. (1951). Inhibition of the rat uterus by adrenergic drugs has also been reported by Miller (1967); Levy and Tozzi (1963); Triner et al. (1970); Triner et al. (1973); Marshall (1973); Diamond (1973). Adrenergic receptors in the rat uterus have been classified as beta-adrenergic receptors although Levy and Tozzi (1963) reported the presence of alpha receptors as well. Levy and Ahlquist (1961) showed that phentolamine could reduce adrenergic effects in the rat uterus. Various other authors have failed to confirm the presence of alpha-receptors in rat uterus (Leonard 1972; Marshall 1970; Kroeger and Marshall 1974). Both histaminergic and adrenergic drugs produce relaxation in the rat uterus. The adrenergic response is blocked by beta-antagonists (Schild 1967; Triner et al. 1969; Szego and Davis 1969; Kroeger and Marshall 1971). However classical antihistamines such as mepyramine do not antagonize the histamine induced relaxation in the rat uterus (Ash and Schild 1966). Black et al. (1972) were able to block the histamine effects on rat uterus by burimamide. They further reported that histamine receptors in rat uterus were similar to those involved in histamine-stimulated gastric secretion. Blyth (1973), reported that the H_2 receptor may be involved in histamine effects on the rat uterus. Tozzi (1973) however reported that histamine-induced inhibition of the rat uterus is similar to that of tyramine and that it indirectly activates beta-adrenergic receptors by releasing catecholamines. Propranolol, an effective beta-adrenergic blocking agent produced a significant parallel shift of the histamine, tyramine and isoproterenol dose-response curves. Jensen and Vennerød (1961) have also previously reported that dichloriso-

proterenol blocked histamine effects on the isolated rat uterus. The findings of Jensen and Vennerod (1961) and Tozzi (1973) suggest that histamine acts either on the same structures as does epinephrine or on a subsequent stage in an epinephrine-initiated process. Tachyphylaxis to histamine and tyramine on rat uterus have been previously reported by Tozzi and Roth (1967). Lack of tachyphylaxis to isoproterenol has been reported by Levy and Tozzi (1963). All of the evidence suggests that the action of histamine on the rat uterus is more like that of tyramine than like that of isoprotenerol. The blockade of histamine by cocaine clearly indicates that the inhibitory action of histamine on the rat uterus as the result of indirect activation of beta-adrenergic receptors through the release of catecholamine (Tozzi and Roth 1967; Tozzi 1973). Green and Miller (1966) reported that the effects of histamine on the rat uterus were blocked in the rats treated with reserpine. If histamine works through the release of catecholamines in the rat uterus, then histamine would be expected to produce both characteristics of beta-adrenergic receptor stimulation. The stimulation of beta-receptors by the catecholamines results in the elevation of cyclic AMP (Triner et al. 1970; Marshall 1973; Marshall and Kroeger 1973; Kroeger and Marshall 1974). The ability of various catecholamines to relax the uterus and to increase its cyclic AMP content is directly related to the effectiveness of these amines as activators of beta-adrenoceptors (Kroeger and Marshall 1974). Both relaxation and the increase in cyclic AMP is prevented by beta-blockers (Triner et al. 1971). Db. cyclic AMP and the phosphodiesterase inhibitor papaverine, relaxed the smooth muscle and potentiated the inhibitory effects of beta-adrenoceptor activation (McFarland et al. 1971; Mitznegg et al. 1970; Pösch and Kukovetz 1967; Polacek and Daniel 1971; Somlyo et al. 1970; Takagi et al. 1971). Histamine activates cardiac adenylate cyclase and

increases cyclic AMP. (McNeill and Muschek 1972, Klein and Levey 1971) and the cardiac effects of histamine are specific and direct. Similarly, histamine also stimulates the gastric mucosa and elevates cyclic AMP (Shaw and Ramwell 1968; Bieck, Oates and Robison 1973). Both cardiac and gastric histamine receptors are classified as H_2 type receptors. (Black et al. 1972). If the histamine receptor in rat uterus is also of H_2 type, then its stimulation by histamine may result in an increase in uterine cyclic AMP. Therefore in the present study the histamine interaction with adenylate cyclase particles prepared from myometrium was investigated as well as the relaxing effect of histamine and its analogs.

IV Histamine-guinea pig ileum interaction:

The specific antagonism of histamine by antihistaminic drugs characterizes one type of histamine receptors for which Ash and Schild (1966) suggested the symbol H_1 . Such receptors occur in guinea pig ileum and bronchi (Arunlakshana and Schild 1959). The histamine H_2 type of receptors such as in the heart and in the gastric mucosa are associated with the enzyme adenylate cyclase (Pösch and Kukovetz 1967; McNeill and Muschek 1972). Stimulation of H_2 -receptors causes the subsequent increase in cyclic AMP (Pösch and Kukovetz 1967; McNeill and Muschek 1972; Bieck, Oates and Robison 1973).

H_1 receptors have not been investigated with regard to their possible involvement with adenylate cyclase. It was therefore of interest to investigate the effects of histamine on such receptors in order to determine if cyclic AMP was involved.

D. Interaction of adrenergic and histaminergic drugs with agents affecting phosphodiesterase

Adrenergic and histaminergic drugs may exert their inotropic and chronotropic effect through elevation of cyclic AMP (Robison et al. 1965; Sutherland et al. 1968; Pösch and Kukovetz 1967; McNeill and Muschek 1972). Increased levels of cyclic AMP may cause an increase in cardiac contractility and phosphorylase a. Blockade of the increase in cyclic AMP by appropriate blocking agents will result in the blockade of cardiac responses. Other criteria to establish that cyclic AMP is involved as a 'second messenger' have been established by Sutherland et al. (1968). For example, the effects of drugs working through cyclic AMP should be enhanced by prior administration of a drug that inhibits the breakdown of the nucleotide by inhibiting phosphodiesterase. The methylxanthines are known to inhibit phosphodiesterase in vitro (Butcher and Sutherland 1962). The influence of theophylline on the cardiac response to catecholamines has been the subject of several studies. Initially, Rall and West (1963), reported that the inotropic effects of norepinephrine in isolated rabbit atria were potentiated in the presence of theophylline. Subsequently, Hess and co-workers (1963) found that theophylline pretreatment reduced the contractile response to epinephrine in isolated perfused rat hearts. The discrepancies in their findings could be due to the different doses of theophylline used. McNeill, Nassar and Brody (1969) established that the dose of theophylline which would potentiate the effects of the norepinephrine on cardiac phosphorylase was cardiodepressant. Similarities have been reported between the cardiovascular effects of the methylxanthines and the β -adrenergic effects of adrenergic amines (Robison et al. 1965). For example, both groups of drugs increase cardiac contractility and relax the intestinal smooth muscle (Pfaffman and

Mcfarland (1973). Papaverine, another phosphodiesterase inhibitor also will produce smooth muscle relaxation (Kukovetz and Pösch 1971). Similar effects have also been reported for catecholamines. Since the positive inotropic response to theophylline was not blocked by propranolol or phentolamine, it was therefore not mediated by catecholamine release (Massingham and Nasmyth 1972). Both normal and reserpinized atria responded with increased concentration in a solution containing caffeine. In recent years, however, a number of investigations emphasized the differences rather than similarities between xanthines and catecholamines (deGubareff and Sleator 1965; McNeill et al. 1969; Blinks et al. 1972). Since methylxanthines have been found to release ionized calcium from intracellular storage sites, this may be the process whereby the xanthines increase myoplasmic calcium ions to cause positive inotropic effects (Nayler 1963); Nayler (1967), demonstrated that the enhanced influx of calcium ions, which accompanies excitation in caffeine-treated toad hearts, resulted in a relatively high myoplasmic calcium ion concentration and hence more powerful contractions. The action of caffeine in producing contractures in skeletal muscle has been studied by Axelsson and Thesleff (1958) and Frank (1962), and it has been shown that this process is intimately associated with the intracellular Ca^{++} concentration. Caffeine causes an increased efflux of Ca^{++} from skeletal muscle when placed in a calcium free solution (Bianchi 1961).

In heart muscle, calcium flux measurements (Niedergerke 1959; Winegrade and Shanes 1962) showed that the inward movement of calcium, which occurs with membrane excitation, is the probable trigger mechanism for the initiation of contraction. Chapman and Niedergerke (1970) have proposed that the slow tension changes in frog heart muscle could be related to accumulation and depletion of stored calcium inside the cell.

Despite the evidence that methylxanthines will inhibit phosphodiesterase (Butcher and Sutherland 1962; McNeill et al. 1973) it has not been established that the use of these drugs results in an increase in cardiac cyclic AMP. Support for the theory that cyclic AMP is involved in the cardiac action of certain drugs has been provided by Kukovetz and Pösch (1967), who observed that, imidazole, a phosphodiesterase stimulator, inhibited the positive inotropic and phosphorylase stimulating effects of isoproterenol. As in the case of theophylline, however there is not much data regarding the effect of imidazole on cardiac cyclic AMP. The recent study of Knope et al. (1973) has raised some interesting questions regarding the cardiac actions of imidazole. These authors have shown that imidazole exerted a positive inotropic effect on isolated atria and on the intact heart. Imidazole does not stimulate adenylate cyclase (McNeill and Muschek 1972), its cardiac effects are not enhanced by aminophylline, and its positive inotropic effects were not blocked by propranolol (Knope et al. 1973). Imidazole thus appears to affect the heart directly by a mechanism other than cyclic AMP.

DeMello et al. reported that the addition of imidazole (500 $\mu\text{g/ml}$) to depolarized heart muscle elicited a contracture. DeMello et al. (1973) further reported that imidazole-induced contractures were largely dependent on extracellular calcium concentration. Contractures induced by imidazole were quickly suppressed in the calcium-free solution. Massingham (1969), also reported that imidazole ($5 \times 10^{-2} \text{M}$) increased the response of the ventricle strip to stimulation. The effects of imidazole on the frequency-force characteristics of isolated left rabbit atria are similar to the actions of cardiac glycosides and calcium (Tuttle and Farah 1962). Farah and Witt (1963) suggest that the site of action of imidazole may be upon tissue calcium or calcium turnover.

It is evident that all the effects of theophylline and imidazole cannot be explained in terms of their actions on phosphodiesterase. In light of the above findings the effects of both imidazole and theophylline on cardiac cyclic AMP, phosphorylase activation and contractility, were studied. The interaction between these agents and norepinephrine or histamine on the above parameters was also studied in an effort to determine if cyclic AMP was involved in the action of these drugs.

Specific goals of the present investigation:

1. To study the cardiac effects of phenylephrine and to compare these with the cardiac effects of norepinephrine.
2. To characterize cardiac histamine receptors by means of a selective histamine (H_2) blocking agent, burimamide.
3. To study the interaction between an H_1 blocking agent promethazine, and the histamine cardiac effects.
4. To determine the interaction between histamine and histamine analogs and burimamide on cardiac, adenylate cyclase.
5. To investigate the effects of histamine and histamine analogs, on rat gastric adenylate cyclase and its interaction with burimamide.
6. To study the interaction of histamine on adenylate cyclase prepared from estrogen primed rat uterus.
7. To investigate the effects of histamine on H_1 receptors (guinea pig ileum) with respect to their association or dissociation from adenylate cyclase.
8. To investigate the cardiac effects of theophylline and the possible interactions of theophylline with histamine and norepinephrine on their cardiac biochemical and mechanical effects..

9. To study the effects of the cardiac phosphodiesterase stimulator, imidazole and its interaction with histamine and norepinephrine on cardiac contractility, phosphorylase a activation and cyclic AMP.

MATERIALS AND METHODS

A. Materials

I Animals

Guinea pigs (500-700 gms) and rats (200-250 gms) of either sex were used throughout the investigation. They received food and water ad. libitum.

II Drugs and Chemicals

The following chemicals and drugs were purchased from Sigma Chemical Co. St. Louis, Mo. Cyclic AMP (phosphoric acid), ATP (disodium salt), protein kinase inhibitor, cyclic AMP dependent binding protein, phenylephrine hydrochloride, 1-norepinephrine HCl (1-arterenol HCl), Imidazole (grade III, crystalline), Glucose 1-phosphate (dipotassium salt), albumin (Bovine), 5', AMP (crystalline), Tris (hydroxymethyl)-Aminomethane, 1-amino-2 naphthol-4 sulfonic acid (purified grade III).

The following drugs were also used in the study. Their source of supply is listed: [³H]-cyclic AMP (ammonium salt, 25 Ci per m mole) Amersham-Searle 2-phosphoenol pyruvate (trisodium salt) and pyruvate, Kinase (rabbit muscle) (Calbiochem), Histamine diphosphate (Mann Research laboratories, New York), Heparin sodium (Nutritional Biochemicals Corporation, Cleveland, Ohio), Propranolol (Ayerst Laboratories, Inc., New York), Betazole HCl and 3-(β-aminoethyl)-1,2,4-Triazole dihydrochloride (Eli Lilly and Company, Indianapolis, Ind.), Burimamide [N-methyl-N'-(4-(4,5)-imidazolyl)butyl Thiourea] (Smith Kline and French Laboratories Ltd., Welwyn Garden City, Herts., England), theophylline (Merck and Company, Inc., Rahway, N.J.), Promethazine

HCl (Poulenc, Montreal, Canada). All other solvents and reagents were AR grade. They were used as they were received without further purification. The chemicals used in the study were obtained from the following sources: Ammonium acetate (crystals), cupric sulphate, Ethylene-dinitrilo tetraacetic acid (EDTA) (disodium salt), Potassium chloride (granular), Sodium carbonate (granular, anhydrous), Sodium tartrate (granular), Sodium sulfide (granular, anhydrous), Sodium bisulfite (granular), Sodium fluoride were all purchased from Mallinckrodt Chemical works. Dowex (anion exchange resin, 100-200 mesh, Bio-Rad laboratories), D-Glucose (BDH), phenol-reagent (Folin and Ciocalteu, Harleco Chemical works), POPOP (1-4,Bis[2-(5 phenyloxazolyl)]-benzene, Sintillation grade), PPO, (2,5-Di-phenyloxazole, Kent Laboratories).

B. Methods

I Phosphorylase Assay:

Phosphorylase was determined by the method described by McNeill and Brody (1966). This was a modification of the original method described by Cori and Cori (1940). The details of the procedure are given below:

From a frozen heart tissue, a 70-100 mg portion of the apex of the heart was homogenized in 200 volumes of solution which contained 0.05M Tris, 0.001M EDTA, 0.02M NaF and 0.3% serum albumin. The tissue homogenate was centrifuged at 10,000 g for 10 minutes. 0.2 ml of the clear supernatant was added to each incubation tube. There were 6 incubation tubes for each heart, divided into two groups, one group of incubation tubes numbered 1, 3, 4 and the other group of incubation tubes numbered 2, 5, 6. The incubation tubes numbered 1, 3, 4 contained tris buffer + (glycogen + GIP) and tubes 2, 5, 6 contained (Tris buffer + 5' AMP) + (glycogen + GIP). Tubes 1 and 2 served

as blanks. The rest of the tubes 3, 4, 5, 6, were incubated at 37°C for 30 minutes for guinea pig hearts and 15 minutes for rat hearts. The reaction was terminated by adding 2.0 ml of 10% trichloroacetic acid to the blank and incubating tubes after placing them in ice.

The tubes were then centrifuged at 15,000 RPM for 10 min. at room temperature. The clear supernatant was added to phototubes to which had been added 6.6 ml of diluted molybdate solution and 0.4 ml of reducing reagent (Fiske and SubaRow 1925). The tubes were allowed to stand for 15 minutes. The inorganic phosphate liberated during the synthesis of glycogen from G-I-P was read in a Coleman Junior spectrophotometer (Colemann Instrument Inc., Maywood, U.S.A.) at 660mμ. Phosphorylase a activity is represented by the amount of inorganic phosphate in the tubes which did not contain 5'-AMP. The total phosphorylase activity is represented by the amount of phosphate liberated in tubes containing 5'-AMP. The results are expressed as percentage phosphorylase a which is:

$$\frac{\text{Enzyme activity without AMP}}{\text{Enzyme activity with AMP}} \times 100.$$

II Cyclic AMP binding assay (Gilman 1970):

a. Tissue extraction for cyclic AMP assay:

A 70-100 mg piece of the frozen tissue cut from the apex of the heart, was homogenized in one ml. of cold 5% trichloroacetic acid. The samples were centrifuged at 15,000 RPM (room temperature) for 10 minutes. To the clear supernatant was added 0.1 ml of 1 N HCl, and extracted 5 times with (5 ml each time) ether. The ether was previously saturated with water. The final traces of ether from the aqueous extract were removed by flushing with nitrogen gas. The sample preparations were lyophilized and the lyophilized

samples were redissolved in 1.5 ml of 100 mM sodium acetate-acetic acid buffer (pH 4.0) and 20 μ l of the sample was used in the cyclic AMP determination. The tissue extract for each heart was done in duplicate.

b. Cyclic AMP standard:

Unlabelled cyclic AMP (phosphoric acid) was dissolved in water to give a solution containing 2.0 p moles/ml. One to fourteen p moles were used routinely in constructing the standard curve.

ICI: Cyclic AMP Binding "Assay-Mix":

The assay mix had three components: 100 mM sodium acetate-acetic acid buffer (pH 4.0), protein kinase inhibitor and [3 H]cyclic AMP.

d. Experimental details:

The reaction was conducted in an ice-bath in a final volume of 200 μ l: Each assay tube contained, 100 μ l of assay mix, which contained: 2 p moles of labelled cyclic AMP, 10-70 μ l, representing 2-14 p moles of unlabelled cyclic AMP, and 30 μ l of cyclic AMP dependent binding protein was added to each assay tube. The equilibrium was reached in 90 min. at 4°C. The mixture was diluted with 1 ml of cold 20 mM K_3PO_4 buffer, pH 6.0. Four to five min later, the contents in the incubation tubes were passed through 24 mm millipore filters (cellulose ester, HAWP 02400, HA, 0.45 μ , white plain), previously rinsed with the same buffer. The tubes were washed with 8.0 ml of cold 20 mM K_3PO_4 buffer. The millipore filters were placed in clean dry glass vials and were dried at 150°F for one hour. To each vial was added 10.0 ml of liquid scintillation cocktail. The radioactivity was determined by scintillation counting in a nuclear chicago isocap/300 model fluid counter.

The efficiency for each sample was read from the quench curve, obtained by plotting Channels ratio against efficiency, given by the quenched [^3H] standard. The CPM recorded for each sample were corrected to DPM. The standard curve for cyclic AMP was a plot of DPM against total p moles of cyclic AMP ([^3H]-cyclic AMP and unlabelled cyclic AMP standard) present in each tube. A straight line relation was obtained on a logarithmic plot between 2 p moles to 14 p moles of total cyclic AMP per tube. Amounts of cyclic AMP in the unknown sample with a given DPM could thus be read from the standard curve.

III Cardiac adenylate cyclase preparation:

A washed particle preparation containing adenylate cyclase was prepared from guinea pig heart by the method of Drummond and Duncan (1970). The details are as follows:

The guinea pig heart was perfused with normal saline to remove all blood. The heart was weighed, cut and homogenized with 10 volumes of tris-HCl buffer for one min. in a Sorvall Omni mixer. The homogenate was passed through a coarse sieve, and centrifuged at 1000 g. for 15 minutes. The supernatant was discarded and the pellet was washed twice with tris buffer. The washed particles were suspended in 5 volumes of tris buffer, and slightly homogenized. The preparations were used within 30 minutes. Imay et al. (1951). The preparations were used within 30 minutes.

IV Gastric adenylate cyclase preparation:

The rats were starved for 24 hours before sacrifice. The whole stomach was removed, washed with normal saline and inverted inside out. The stomachs were then homogenized in 10 mM tris HCl for one min. in a sorvall omni-mixer. The homogenate was passed through a coarse sieve and centrifuged

at 1000 xg for 15 min. at 4°C. The pellet was washed twice with tris buffer. The supernatant was discarded and pellet was resuspended and slightly homogenized in a hand glass homogenizer.

V Uterine adenylate cyclase preparation:

Female wistar rats weighing 200-250 gms, were injected with diethyl stilboestral (dissolved in peanut oil, 60 µg/100 gms interaperitoneally) approximately 24 hours before sacrifice. The animals were killed by a blow on the head, the uteri were removed, weighed and homogenized in 10 mM tris HCl. The procedure for preparing the adenylate cyclase particles was identical to that described for cardiac or gastric adenylate cyclase.

VI Adenylate cyclase assay:

Each assay tube (in a total volume of 150 µl) consisted of, tris HCl, 0.3M, theophylline 0.06M, MgSO_4 0.225M, KCl 0.083M, phosphoenol pyruvate 0.3M, pyruvate kinase (1:5 dilution) and ATP 5 mM. After addition of all the components except enzyme, the assay tubes were preincubated for 4 minutes. The reaction was started by adding 50 µl of the enzyme preparation. Incubation was carried at 37°C for 10 min. The reaction was terminated by placing the tubes in a boiling water bath for 3 min. Denatured proteins were removed by centrifugation at 10,000 g for 5 min. A portion of the clear supernatant (50 µl) was diluted 11-fold with 50 mM sodium acetate buffer. A 50 µl aliquot of the sample was used for the determination of cyclic AMP by the method of Gillman (1970). Enzyme activity was expressed in p moles cyclic AMP produced mg^{-1} protein min^{-1} .

VII Determination of protein:

Protein content of the adenylate cyclase was determined by the Lowry method (1951) as described by Sutherland et al. (1949) and modified by Robson et al. (1968).

CHEMICALS AND REAGENTS

Alkaline copper sulfate solution was prepared by adding 1 ml of 2% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1 ml of 4% (w/v) sodium tartrate, diluted to 100 ml with 4% Na_2CO_3 solution. The phenol reagent was prepared by diluting 2N folin-ciocalteau solution (1 part of reagent + 3 parts of distilled water). Protein standard solutions were made from Bovine serum albumin in a concentration range of 10 to 200 $\mu\text{g/ml}$. The standard protein solutions were kept frozen at -2°C .

PROCEDURE

The adenylate cyclase washed particles, were diluted 1:10 in distilled water to place the protein concentration within the range of the albumin standards. Five ml of the alkaline copper solution was added to 0.5 ml of the diluted suspension. This was mixed instantaneously and was placed on a water bath kept at 45°C for 12 min. After exactly 12 min., 0.5 ml of the phenol reagent was added. The contents of the tube were vortexed for 10 sec. and the tubes were left at room temperature for 15 min. The absorbance, relative to a water blank, was then determined by use of a perkin-elmer (Coleman 124) double beam spectrophotometer. Standard albumin solutions were treated in the same manner in order to generate a standard curve from which the unknown protein concentrations were estimated.

VIII Time-response study of the agonists:

Guinea pigs (500-700 gm) or rats (200-300 gm) of either sex were

injected with heparin sodium (8 mg/kg SC, in guinea pigs and 2.8 mg/kg SC for rats), ninety minutes prior to sacrifice. The animals were stunned by a blow to the head and the heart was rapidly removed and perfused with Chenoweth-Koelle solution (1946), by the Langendorff technique. The composition of the perfusion fluid has been previously described. The hearts were perfused at a flow rate of 4 ml for both species per min. at 37°C. The flow rate was maintained by means of a Holter-microinfusion roller pump (Extracorporeal Medical specialties, King of prussia, PA, model RL175). Cardiac contractility was monitored by means of a palmer clip placed at the apex of the heart and connected to a Grass force-displacement transducer and recorded on a Grass model 7 polygraph. Diastolic tension was adjusted to 2.0 gm. The hearts were allowed to equilibrate for 15 min. prior to the injection of the agonist. The agonist was injected by means of a side arm cannula. At various time-intervals following the injection the hearts were frozen by means of a pair of Wollenberger tongs (Wollenberger et al. 1960) previously chilled in 2 methylbutane cooled in an alcohol-dryice mixture. The hearts were stored at -80°C, until assayed for cyclic AMP and for phosphorylase activation.

IX Dose-response study of the agonists:

Complete dose-response curves of the agonists on cardiac force of contraction were carried out. From these studies the dose of the agonist which produced the maximum effect was selected for studying the biochemical effects. The times at which the phosphorylase a and cyclic AMP reached a peak level, was used to study the Dose-response effects. The dose was injected by the side arm cannula into the isolated perfused guinea pig or rat heart. The hearts were frozen as described above at 38-40 sec. for phosphorylase measurements and at 16-18 sec. for the measurement of cyclic AMP.

The hearts were stored at -80° until analysed.

X Dose-response study of histamine and its analogs on rat uterus:

The rats were estrogen-pretreated with diethylstilboesterol dissolved in peanut oil (60 μ g/100 gm interaperitoneally) approximately 24 hours before sacrifice. The animals were killed by a blow on the head, and uteri were removed and placed in a NaCl-tris buffered solution (Diamond 1973) at 37°C for 30 minutes prior to suspension in 50 ml organ baths. All buffer solutions were aerated with 100% oxygen.

After 30 minutes, paired segments from one uterine horn were suspended by means of a small stainless steel hooks in organ baths containing the bathing media at 37°C . Tension was measured isometrically by means of a force transducer (Grass FT03C) and recorded on a Grass model 79 polygraph. Tension was slowly increased until the uterus was contracting spontaneously with a resting tension of approximately 0.2-0.5 gm. The uteri were left until these contractions became regular. The bathing medium was then changed to KCl-tris buffered solution. This caused a rapid depolarization of the muscle, and a sustained contraction quickly developed. After 15 minutes exposure to the new bathing medium, histamine, 4-methylhistamine TD and betazole in a 1.0 ml volume was added to the organ bath and relaxation was monitored.

C. Statistical analyses

Statistical analyses were carried out using the 't' test for the unpaired data for cyclic AMP and phosphorylase experiments and 't' test for a paired data for cardiac contractility, rat uterus relaxation and adenylate cyclase activation (Lewis 1966). The results were calculated using Wange #600, calculator using programme #9, and 10 for 't' test. A probability level of

0.05 was chosen as indicating significance.

RESULTS

A. Norepinephrine and phenylephrine cardiac effects

A complete dose-response study with phenylephrine and norepinephrine on cardiac contractility revealed that 1.0 mg of phenylephrine or 1.0 μ g of norepinephrine produced the maximum positive inotropic effect. Maximum effective doses of agonists were used in the time-response studies. Fig. 5 illustrates the data obtained from a study of the effect of time on the norepinephrine (1 μ g) response. Norepinephrine produced a positive inotropic response, an increase in phosphorylase a and cyclic AMP elevation. As a result of the norepinephrine injection, cyclic AMP levels were elevated significantly ($P < .05$) from the control value of $0.52 \pm .04$ nmoles/gm wet wt. at six seconds. The rise in cyclic AMP was very sharp, reaching nearly a 5 fold increase over the control value in 14 seconds. Significant elevations in contractility and phosphorylase a in response to norepinephrine were observed at 11 sec. Contractility increased to a maximum of $85.0 \pm 1.0\%$ over control (20 sec.). Control phosphorylase a values in the experiments were $5.6 \pm 1.8\%$ and norepinephrine increased the phosphorylase a values to a peak of $55.0 \pm 1.0\%$ at 38 sec.

Similarly 1.0 mg of phenylephrine increased all three parameters. Fig. 6 shows the effect of time on the phenylephrine responses. Cyclic AMP levels were significantly different from control values of 0.40 ± 0.12 nmoles/gm wet wt. at 12 sec. following phenylephrine injection and reached a peak at 18 sec. Cardiac contractility was elevated at 15 sec. and peaked at 20-25 sec. Phosphorylase a was significantly increased at 18 sec. and peaked at 30-40 sec. The maximum cyclic AMP value obtained with phenylephrine was 1.3 nmoles/gm wet wt. while contractility increased to $42.5 \pm 1.5\%$ over control and phosphorylase a increased to $35.0 \pm 1.0\%$. All values were significantly less than those

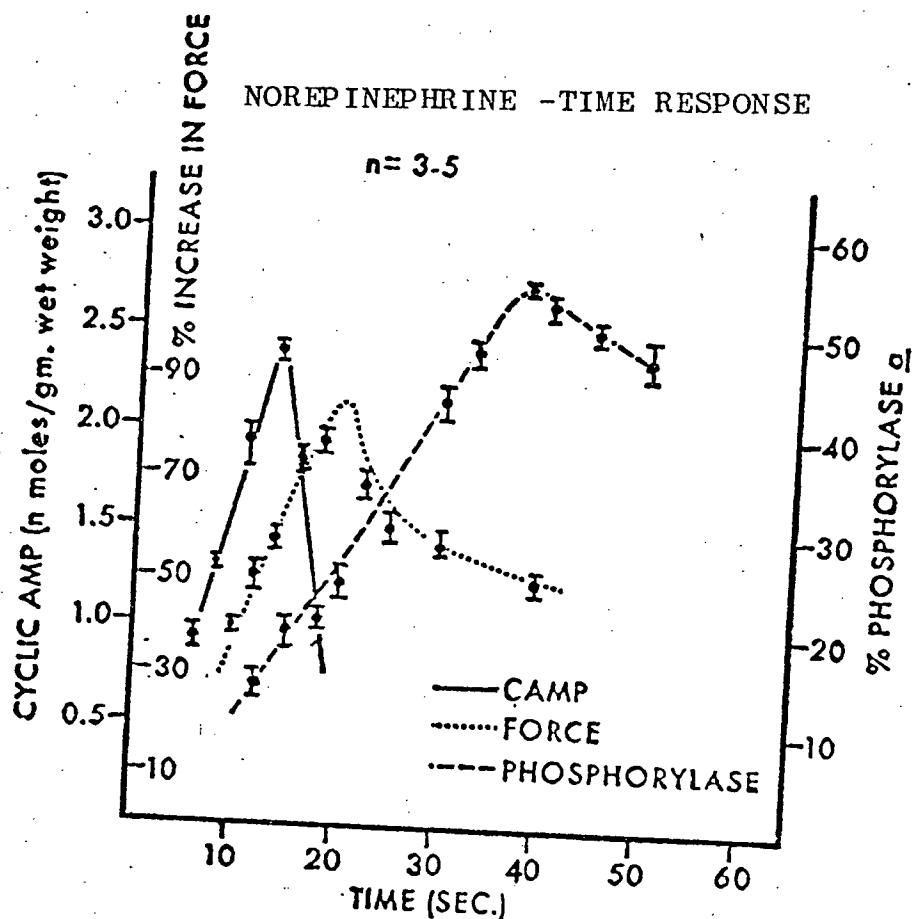


FIG. 5. Time-response effects of norepinephrine ($1 \mu\text{g}$) on cardiac cyclic AMP, percent phosphorylase a and contractility in the perfused guinea pig heart. Each point represents the mean of three to five hearts and the vertical bars represent \pm S.E.M.

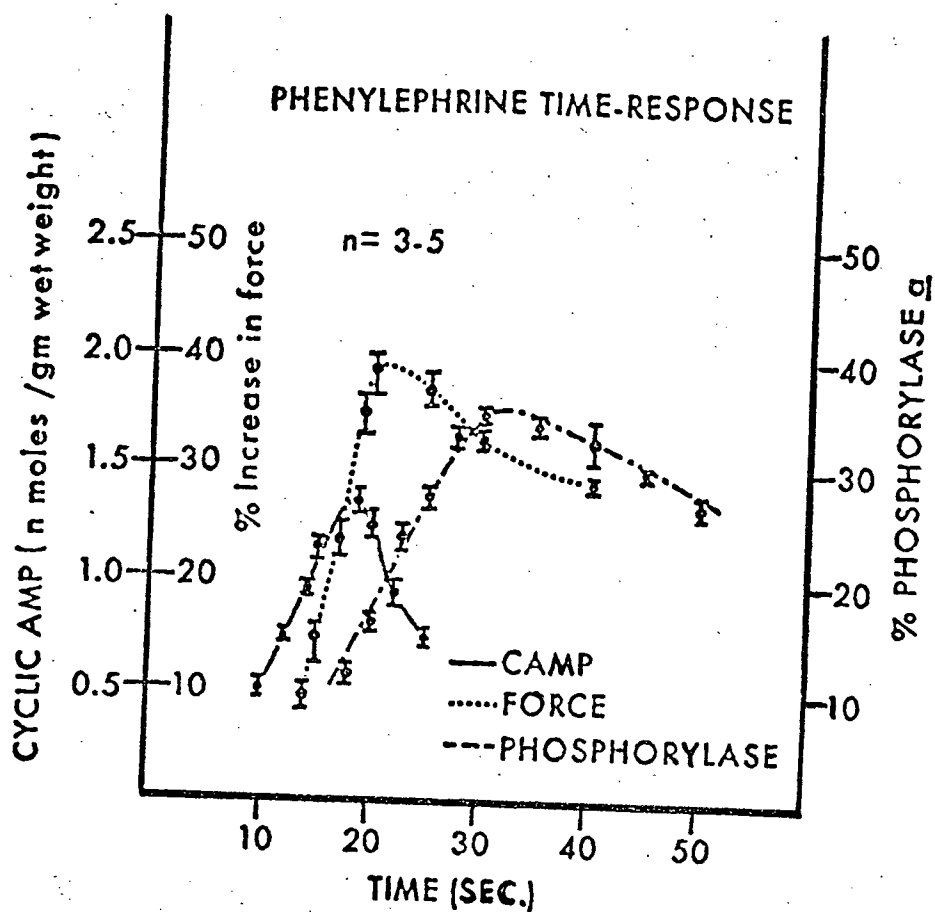


FIG. 6. Time-response effects of phenylephrine (1 mg) on cardiac cyclic AMP, percent phosphorylase α and contractility in the perfused guinea pig heart. Each point represents the mean of three to five hearts and the vertical bars represent \pm S.E.M.

obtained with norepinephrine. In the next experiment, a dose-response relationship of phenylephrine and norepinephrine in their abilities to increase cardiac cyclic AMP was studied (Fig. 7). From the time-response study, the time at which cyclic AMP peaked was selected for a dose-response study. Different doses of the amines were injected and hearts were frozen and analysed for cyclic AMP. The data demonstrate that both amines produce dose-dependent increases in cyclic AMP. Maximum values of cyclic AMP obtained with norepinephrine were 2.3 ± 0.43 nmoles/gm wet wt. and with phenylephrine were $1.25 \pm .101$ nmoles/gm wet wt. Phenylephrine was less potent and less effective than norepinephrine in elevating cardiac contractility, phosphorylase a and cyclic AMP.

B. Histamine and histamine analogs cardiac effects

A dose-response study with histamine, TD and betazole on cardiac contractility (Fig. 8) showed that histamine was more potent than its two analogs. From Fig. 8, the dose for histamine, TD and betazole was selected to study their time-response relationship. Fig. 9, 10, 11, shows that histamine TD and betazole all increased cyclic AMP, phosphorylase a and cardiac contractility. Significant elevation ($P < .05$) of histamine-induced cyclic AMP was observed at 11 sec., peaking at 18-19 sec. Contractility and phosphorylase a values were significantly elevated at 15 sec. The control values of cyclic AMP were $0.50 \pm .08$ nmoles/gm wet wt. The increase in cyclic AMP was five fold over the control values. Cardiac contractility peaked at 20-30 sec. while phosphorylase a values peaked at a value of $41.0 \pm 1.0\%$ at a later time (30-40 sec.).

Following an injection of TD in the isolated guinea pig heart, cyclic AMP levels increased from 0.5 ± 0.1 nmoles/gm wet wt. to 2.0 ± 0.10 nmoles/gm

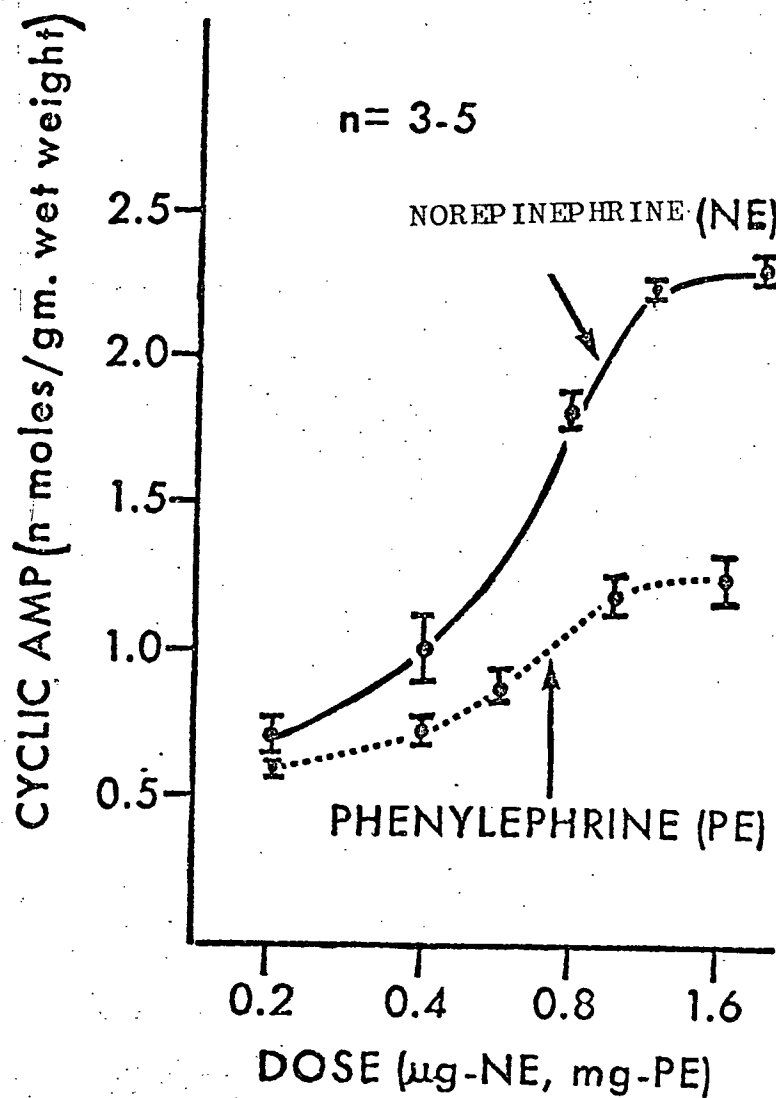


FIG. 7. Dose-response effect of norepinephrine and phenylephrine on cardiac cyclic AMP in the perfused guinea pig heart. Hearts were frozen at the peak of the elevation of cyclic AMP (14 seconds with norepinephrine and 18 seconds with phenylephrine). Each point represents the mean of three to five hearts and the vertical bars represent \pm S.E.M.

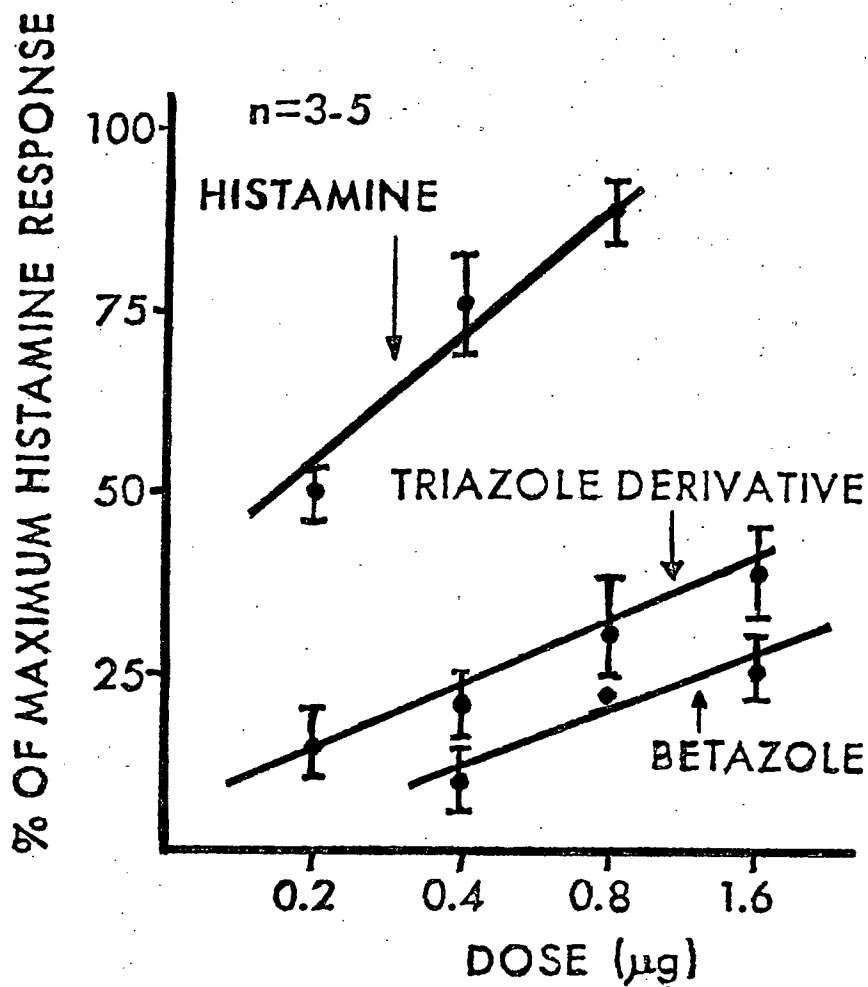


FIG. 8. The effect of histamine, triazole derivative and betazole on cardiac contractility on the isolated, perfused guinea pig heart. Each point represents the mean \pm S.E.M. of three to five determinations. Results are expressed as a percentage of the maximum response that could be obtained with histamine.

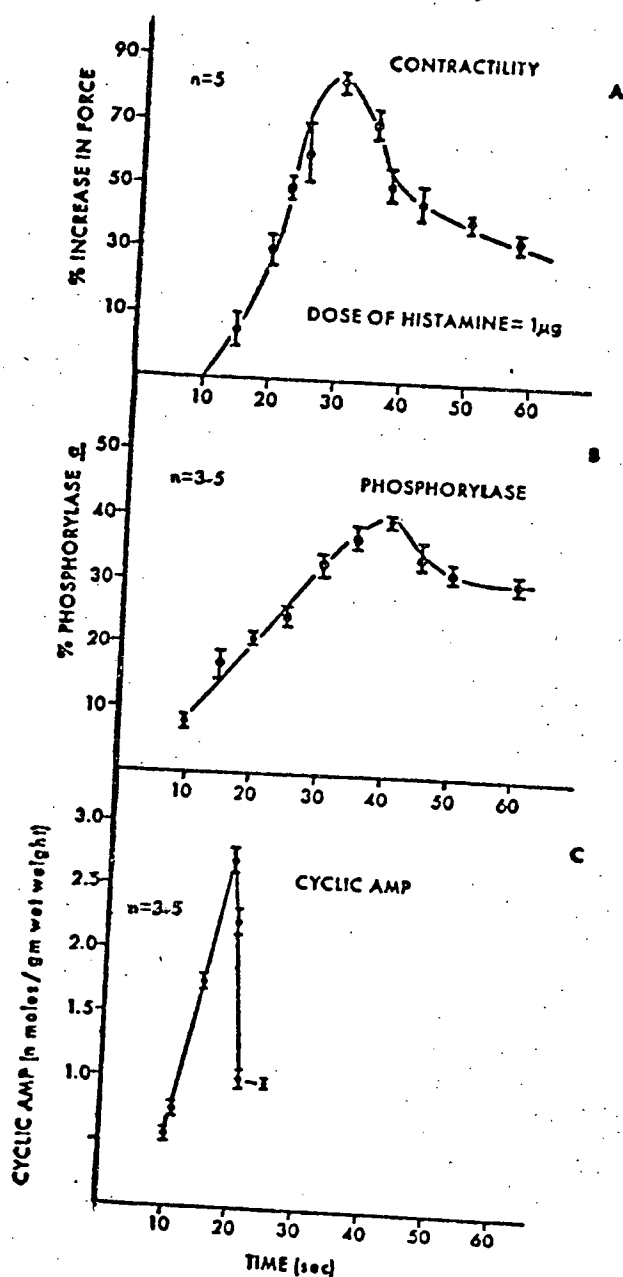


FIG. 9. The effect of time on the ability of $1\mu\text{g}$ of histamine to elevate (A) contractility, (B) phosphorylase and (C) cyclic AMP in the isolated, perfused guinea pig heart. Cyclic AMP was significantly elevated ($P < .05$) at 11 seconds. Contractility and phosphorylase a values were significantly elevated at 15 seconds. Control cyclic AMP levels were 0.50 ± 0.08 nmol/g wet wt. of tissue. Control percent phosphorylase a levels were $5.0 \pm 1.0\%$.

wet wt., a four fold increase over the control values. Cardiac force of contraction and phosphorylase a levels were significantly increased ($P < 0.05$) at 15 and 25 sec. respectively. TD-induced increases in contractility reached a peak at 20-30 sec., but phosphorylase a elevation lagged behind, reaching a peak at 40 sec. Contractility increased to $48.2 \pm 1.8\%$ over control, while phosphorylase a values peaked at a value of $17.5 \pm 2.0\%$ at a later time. Fig. 11 shows the time-response correlation of betazole-induced changes in cyclic AMP, contractility and phosphorylase a values. Cyclic AMP reached a peak at 20 sec., contractility and phosphorylase a reached a maximum at 20-30 sec. and 40 sec. respectively.

The effect of these agonists on cyclic AMP formation was dose-dependent as shown in Fig. 12. The order of potency in increasing cyclic AMP was histamine > TD > betazole. Peak values of cyclic AMP for histamine were 2.3 ± 0.05 , for TD 2.1 ± 0.1 and for betazole 1.5 ± 0.075 nmoles/gm wet wt. of the tissue.

In the next series of experiments, the interaction of burimamide with histamine, TD and betazole on cardiac contractility, phosphorylase a activation and cyclic AMP was studied. Fig. 13 shows the effect of various concentrations ($2-16 \times 10^{-5}$ M) of burimamide on the contractile response to histamine. The data were plotted as a percentage of the maximum response that could be attained with histamine. The next two Figs. 14, 15, show the effect of 0.5×10^{-5} M and 1×10^{-6} M burimamide on the contractile response produced by TD and betazole. The dose-response curves of histamine, TD and betazole on cardiac contractility were shifted in an apparently parallel fashion by the doses of burimamide used. Similar effects were noted with burimamide in shifting the dose-response curve of histamine, TD and betazole on cardiac phosphorylase activation (Fig. 16, 17, 18).

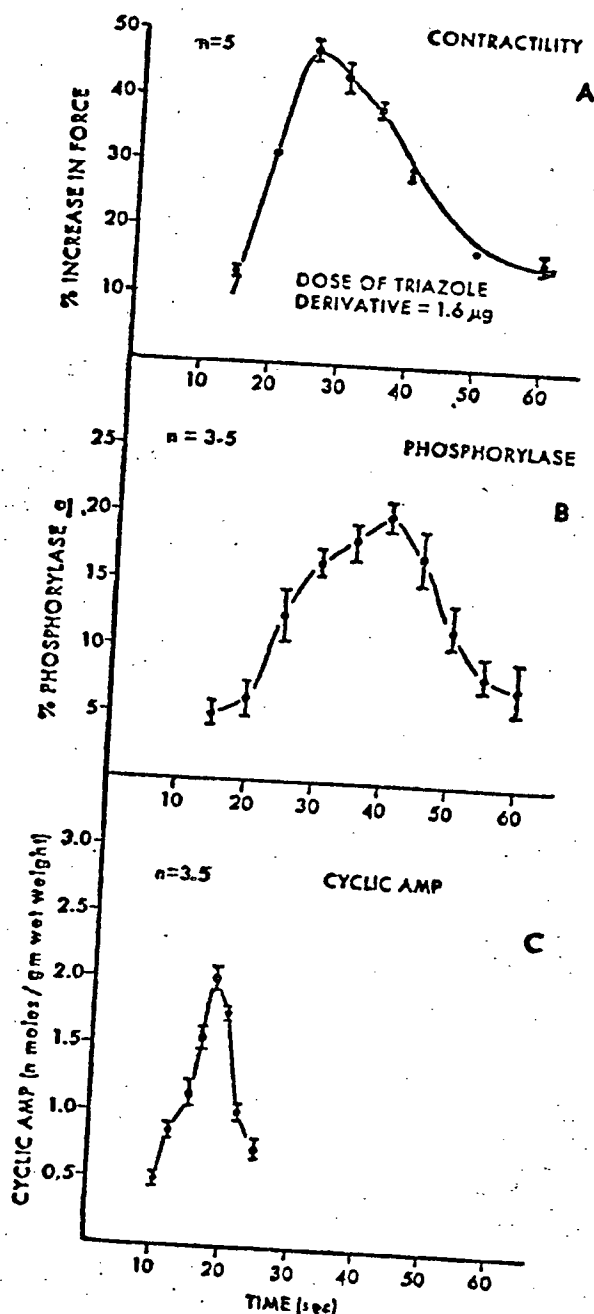


FIG. 10. The effect of time on the ability of $1.6 \mu\text{g}$ of triazole derivative to elevate (A) contractility, (B) phosphorylase and (C) cyclic AMP in the isolated, perfused guinea pig heart. Cyclic AMP was significantly ($P < .05$) elevated at 11 seconds, contractility at 15 seconds and phosphorylase at 25 seconds. Control cyclic AMP levels were 0.50 ± 0.10 nmol/g wet wt. of tissue. Control percent phosphorylase a levels were $5.2 \pm 1.0\%$.

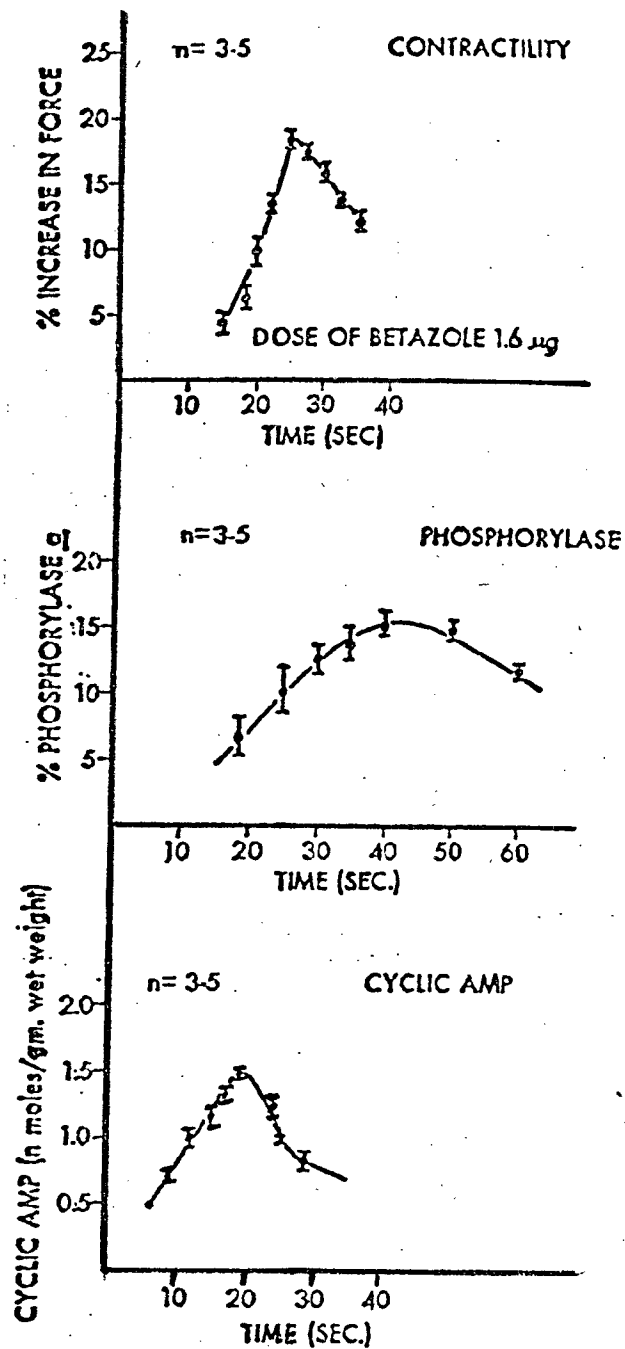


FIG. 11. The effect of time on the ability of 1.6 μg of betazole to elevate (A) contractility, (B) phosphorylase and (C) cyclic AMP in the isolated, perfused guinea pig heart.

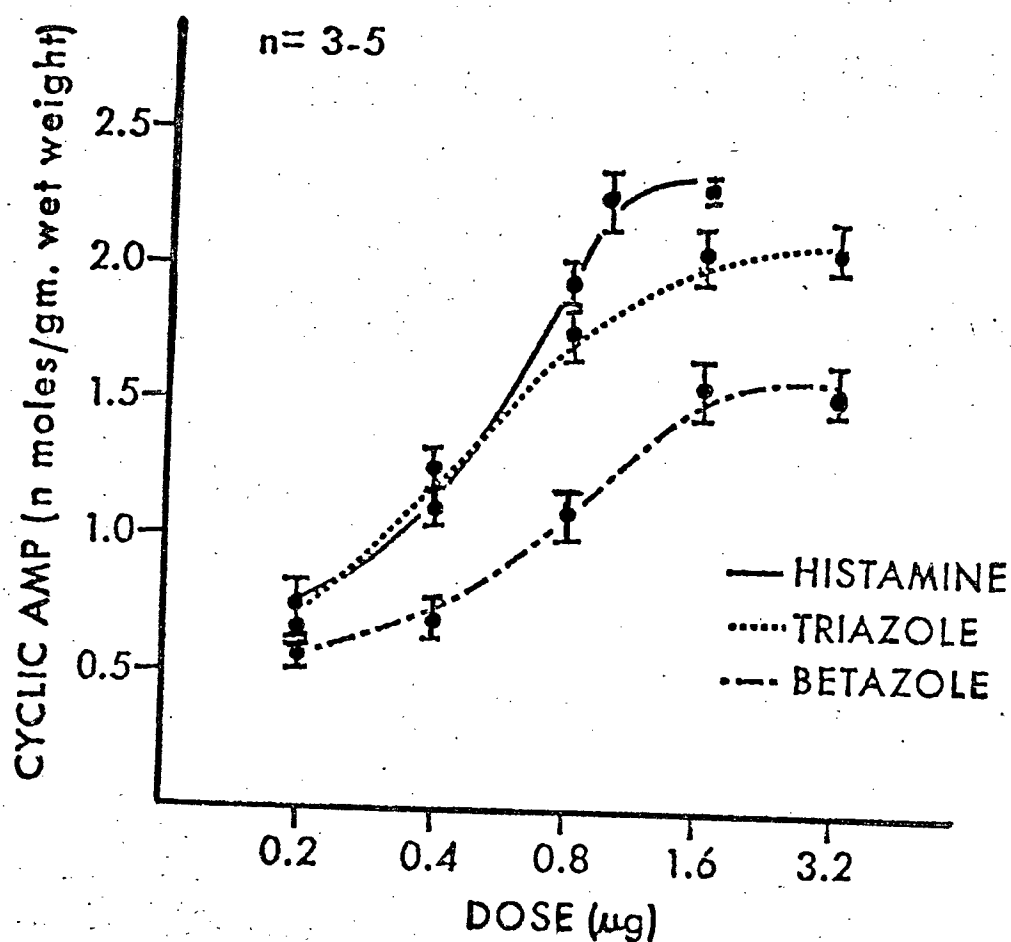


FIG. 12. The effect of histamine, triazole derivative (TRIAZOLE) and betazole on cyclic AMP in the isolated, perfused guinea pig heart. Agonists were injected and the hearts were frozen 18 seconds later at the peak of the response. Control cyclic AMP levels were 0.50 ± 0.09 nmol/g wet wt. of tissue.

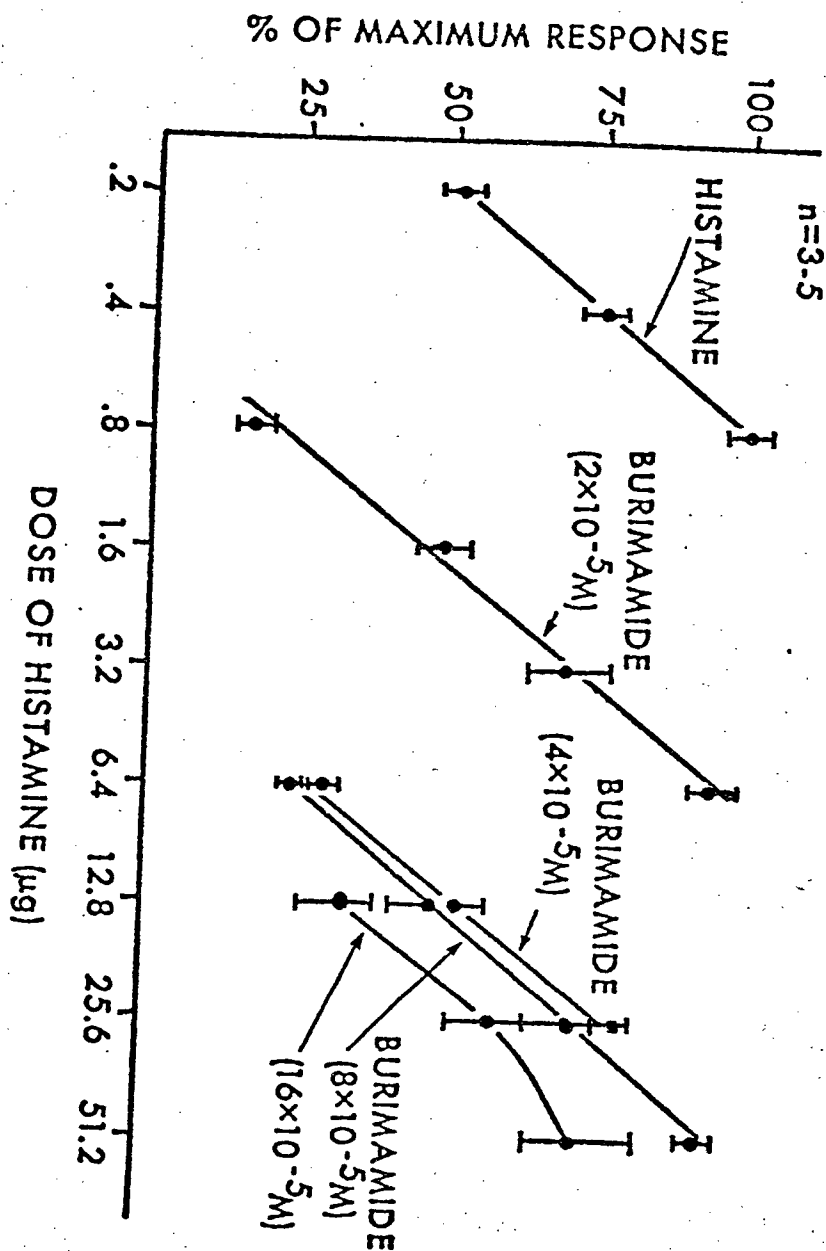


FIG. 13. The effect of various concentrations ($2-16 \times 10^{-5}M$) of burimamide on the contractile response to histamine in the isolated, perfused guinea pig heart. The data are plotted as a percentage of the maximum response that could be attained with histamine.

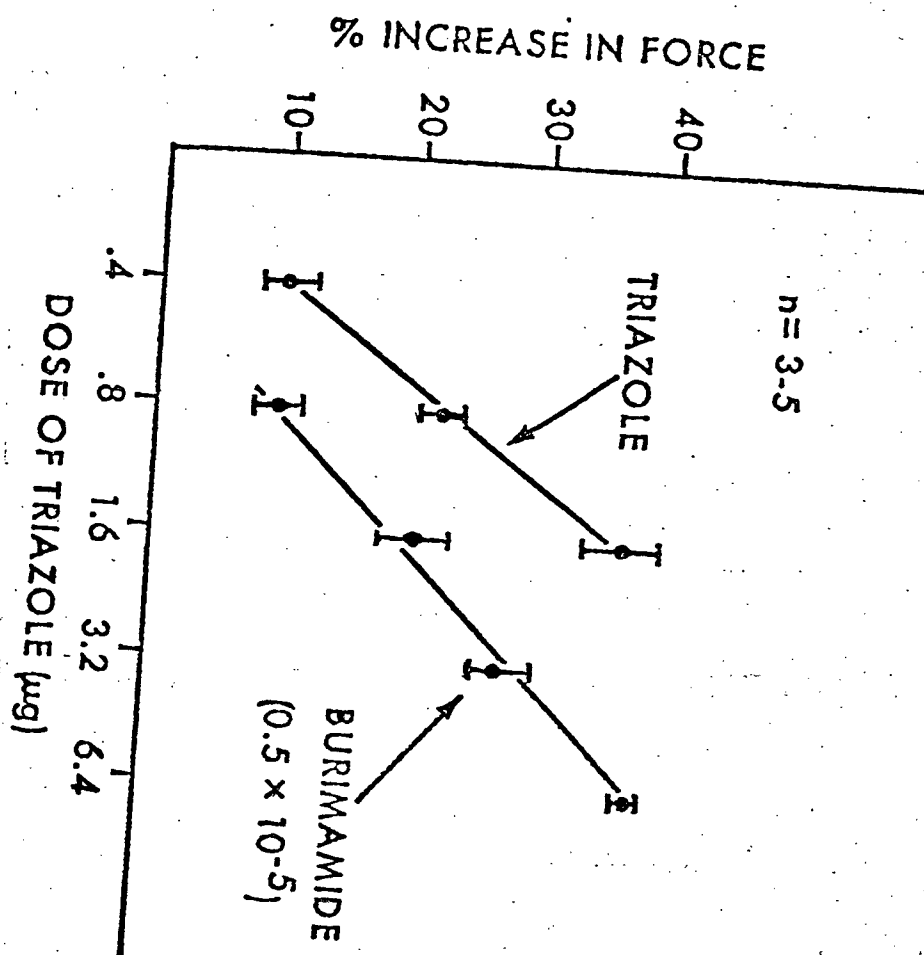


FIG. 14. The effect of burimamide ($0.5 \times 10^{-5} M$) on the contractile response to triazole in the isolated, perfused guinea pig heart. Data are presented as percent increase over control.

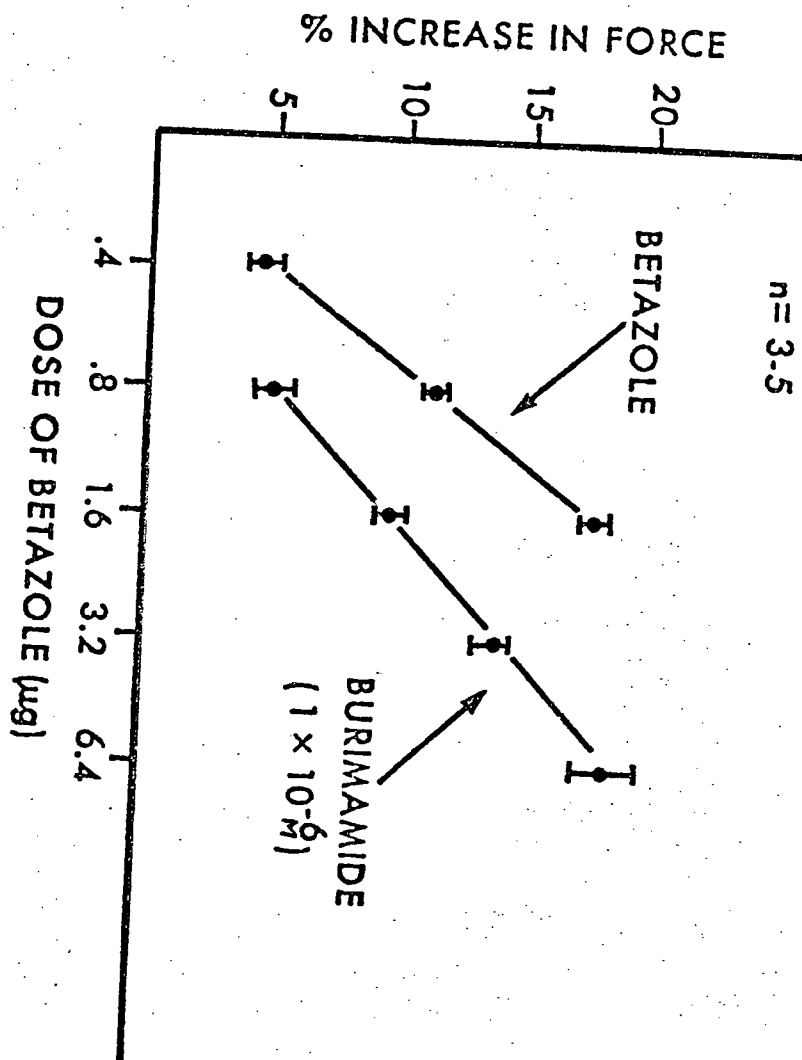


FIG. 15. The effect of betazole and betazole plus burimamide on cardiac phosphorylase activation in the isolated, perfused guinea pig heart. Experiments were performed as described in figure 2.

Histamine produced an increase in cyclic AMP and Fig. 19 illustrates that 2×10^{-5} M burimamide completely abolished the histamine (1 μ g)-induced cyclic AMP elevation. The blockade was overcome by increasing the dose of histamine to 6.4 μ g. The effects of TD (1.6 μ g) and betazole (1.6 μ g) on cardiac cyclic AMP were also blocked by burimamide (Table 3).

The specificity of burimamide in blocking the histamine effects was tested in the next experiments. The data presented in Table 4 illustrates that burimamide (2×10^{-5} M) did not affect the norepinephrine-induced increase in contractility, phosphorylase a or cyclic AMP. Propranolol, a beta-antagonist, did not decrease the histamine-induced biochemical and mechanical effects. In other words, propranolol blocked the effects of norepinephrine and burimamide blocked the effects of histamine and its analogs.

Theophylline (10^{-3} M) potentiated the effects of TD and betazole on cardiac contractility in the isolated perfused guinea pig heart. Table 5 represents the responses due to various doses of TD and betazole on cardiac contractility and their potentiation by theophylline. McNeill and Muschek (1972), had previously reported that theophylline (10^{-3} M) potentiated the histamine effects on cardiac contractility in the isolated perfused guinea pig heart.

Burimamide shifted the dose-response curves for the cardiac effects of histamine to the right. The effects of promethazine (an H_1 receptor antagonist) and the nature of its interaction with cardiac histamine receptors were investigated. Promethazine perfusion in a concentration of $2-4 \times 10^{-6}$ M through the guinea pig heart caused a slight positive inotropic effect (Fig. 20). These effects were not seen at higher promethazine concentrations ($8-16 \times 10^{-6}$ M). Promethazine ($2-4 \times 10^{-6}$ M) produced a positive chronotropic effect but a slight negative inotropic effect was noted at higher concentrations

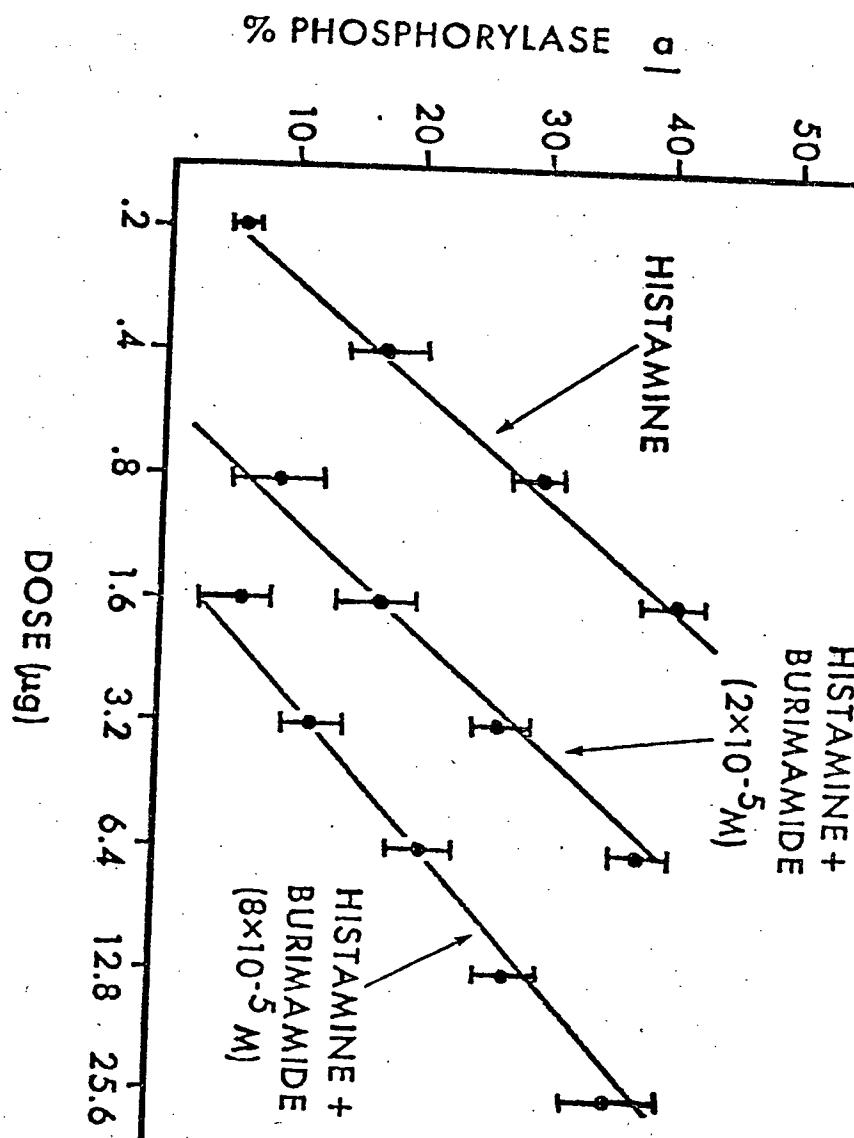


FIG. 16. The effect of histamine and histamine plus burimamide on cardiac phosphorylase activation in the isolated, perfused guinea pig heart. Each point represents the mean percent phosphorylase \pm S.E.M. of five determinations. Burimamide was perfused through the heart for 15 to 20 minutes before histamine injection. Percent phosphorylase α values in hearts injected with buffer solution were $5.0 \pm 1.0\%$.

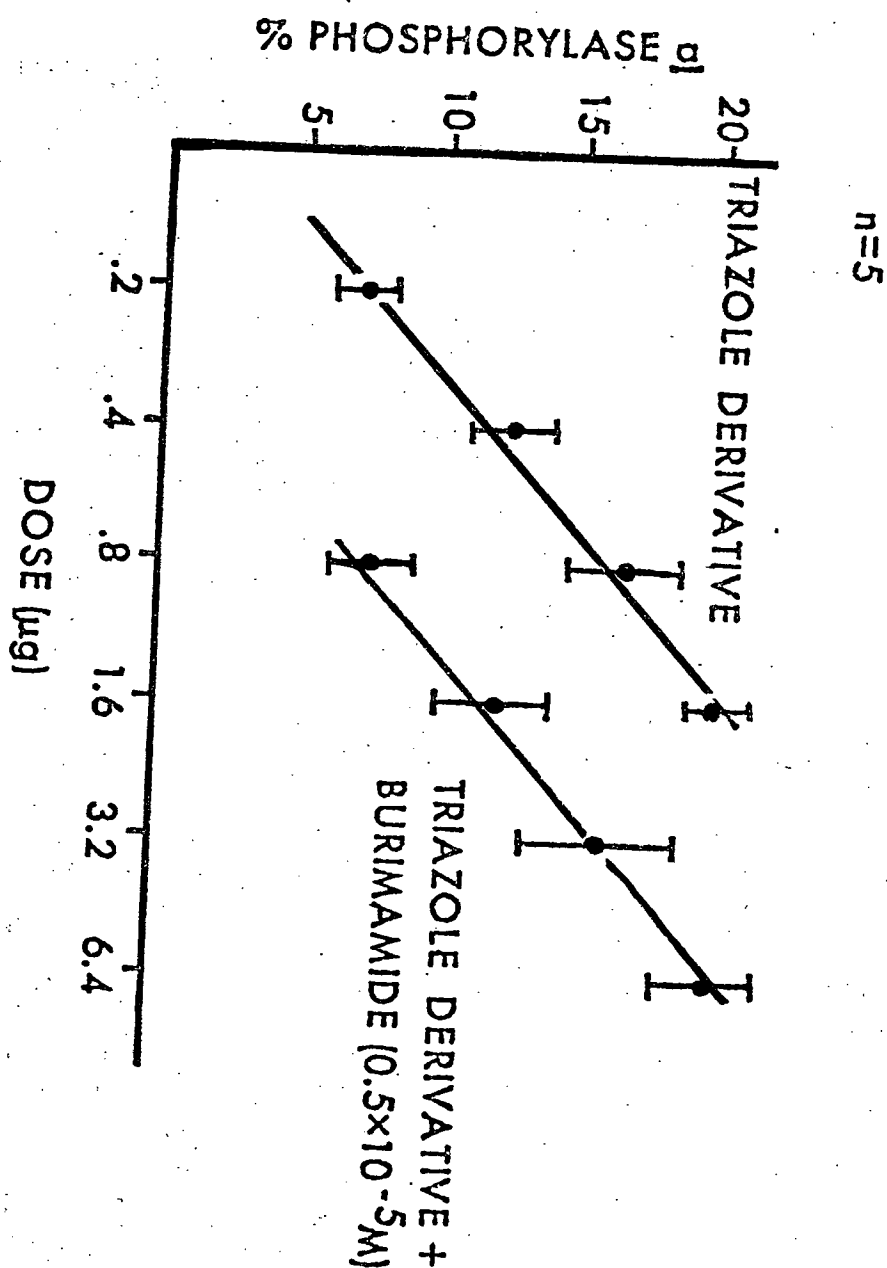


FIG. 17. The effect of triazole derivative and triazole derivative plus burimamide on cardiac phosphorylase activation on the isolated, perfused guinea pig heart. Experimental values are means \pm S.E. of 5 experiments.

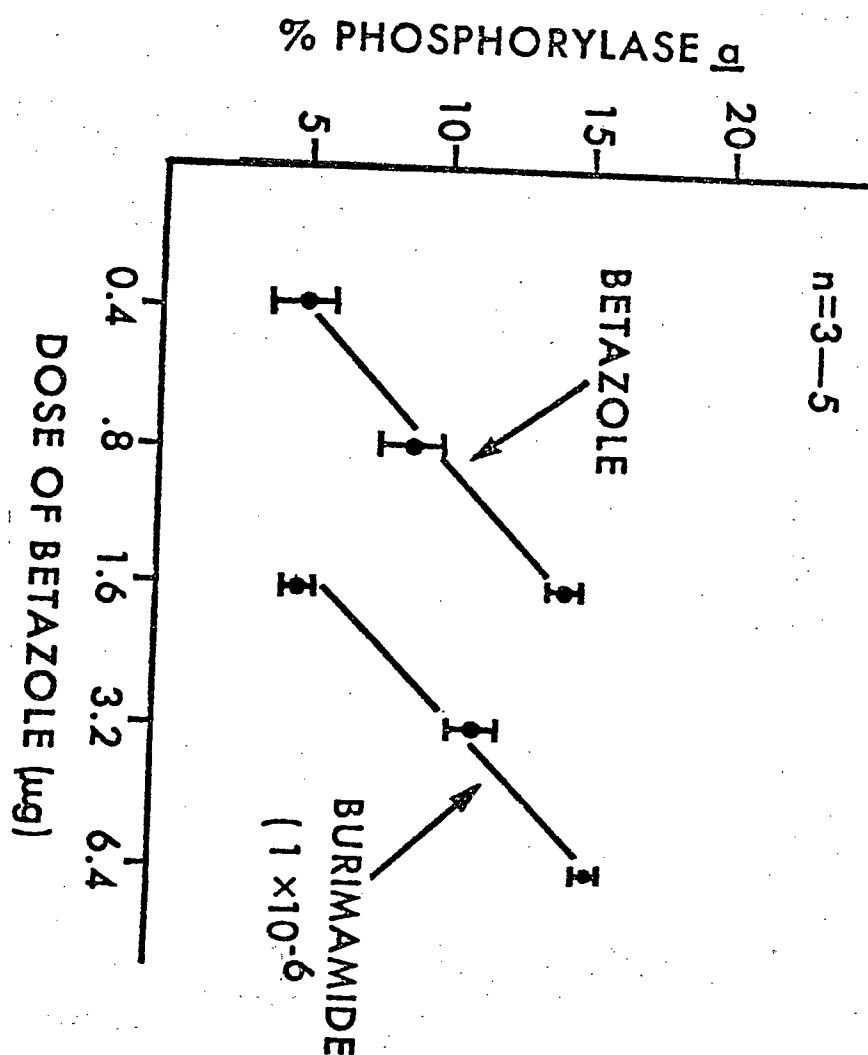


FIG. 18. The effect of burimamide (1×10^{-6} M) on the contractile response to betazole in the isolated, perfused guinea pig heart. Data are presented as percent increase over control.

TABLE 3

Effect of TD, betazole and TD or betazole plus burimamide on cardiac cyclic AMP. Hearts were frozen at the peak of the cyclic AMP response.

Drug Treatment	Burimamide Concentration	Cyclic AMP (nm/gm net weight)
None	0	0.48 ± 0.13
TD 1.6 µg	0	1.97 ± 0.12*
TD 1.6 µg	0.5 x 10 ⁻⁵ M	0.51 ± 0.08
TD 6.4 µg	0.5 x 10 ⁻⁵ M	2.10 ± 0.03*
Betazole 1.6 µg	0	1.38 ± 0.15*
Betazole 1.6 µg	1 x 10 ⁻⁶ M	0.53 ± 0.05
Betazole 6.4 µg	1 x 10 ⁻⁶ M	1.42 ± 0.08*

N = 3-5

* Significantly (P < 0.05) greater than no drug treatment.

TABLE 4

The effect of histamine (1 μ g) and norepinephrine (1 μ g) and the interaction of these drugs with burimamide (2×10^{-5} M) and propranolol (10^{-6} M) on cardiac contractility, phosphorylase and cyclic AMP levels in the perfused guinea pig heart. Histamine and norepinephrine were injected via a side-arm cannula. The antagonists were perfused through the heart for 15-20 min. prior to the injection of the agonist. Cyclic AMP was measured 18 sec. and phosphorylase a 40 sec. after injection of the agonist.

Drug	Cyclic AMP (nmole/gm wet weight)	% Phosphorylase <u>a</u>	Contractility (% increase over control)
Buffer solution	0.68 \pm 0.05	5.0 \pm 1.0	0.00
Histamine	2.50 \pm 0.09 ^a	44.7 \pm 1.2 ^a	90.6 \pm 1.2 ^a
Histamine & Burimamide	0.65 \pm 0.04 ^b	7.5 \pm 1.4 ^b	15 \pm 1.6 ^b
Histamine & Propranolol	2.32 \pm 0.12 ^a	38.4 \pm 2.2 ^a	82.9 \pm 2.3 ^a
Norepinephrine	2.30 \pm 0.12 ^a	57.9 \pm 1.4 ^a	89.0 \pm 1.8 ^a
Norepinephrine & Propranolol	0.68 \pm 0.03 ^c	4.7 \pm 1.2 ^c	9.5 \pm 0.5 ^c
Norepinephrine & Burimamide	2.05 \pm 0.10 ^a	55.2 \pm 1.5 ^a	81.1 \pm 1.5 ^a

a. Significantly increased when compared to buffer solution injected hearts (P < 0.05).

b. Significantly decreased when compared to histamine injected hearts.

c. Significantly decreased when compared to norepinephrine injected hearts.

Each value represents the mean \pm S.E.M. of 3-5 determinations.

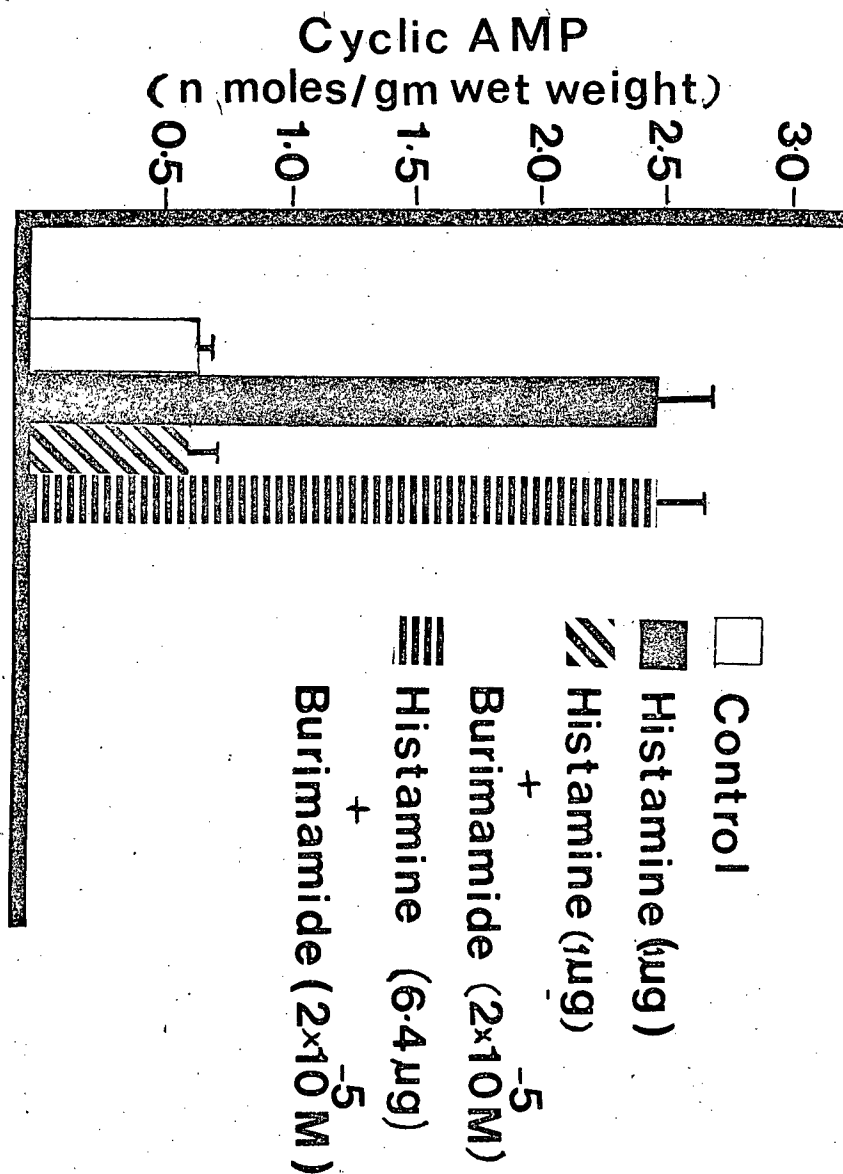


FIG. 19. The effect of histamine and histamine plus burimamide on histamine-induced increases in cyclic AMP. Each bar represents the mean \pm S.E.M. of three to five cyclic AMP determinations. Cyclic AMP was determined in hearts frozen 18 seconds after the injection of histamine.

TABLE 5

Effect of TD and betazole and TD or betazole and theophylline ($10^{-3}M$) on cardiac contractility in the isolated perfused guinea pig heart. Drugs were injected or perfused as described in the Methods. Results are expressed as a % of the maximum response obtained with histamine (1 μg).

Drug Treatment	Dose (μg)			
	0.2	0.4	0.8	1.6
TD	8.6 \pm 2.9	20.0 \pm 2.0	27 \pm 1.0	40.0 \pm 4.3
TD + Theophylline	13.0 \pm 1.0	27.0 \pm 2.0 ^a	39.0 \pm 2.5 ^a	—
Betazole	0.0	10.0 \pm 1.0	19.0 \pm 2.0	25.0 \pm 2.5
Betazole & Theophylline	10.5 \pm 2.3 ^a	17.5 \pm 1.9 ^a	25.0 \pm 2.0 ^a	—

N = 5

a. \pm Significantly (P < 0.05) greater than drug treatment without theophylline.

($8-16 \times 10^{-6} \text{ M}$) as shown in Fig. 21.

Histamine dose-response curves of cardiac contractility and heart rate were obtained in the presence and absence of promethazine ($4-16 \times 10^{-6} \text{ M}$). As seen in Fig. 22 promethazine decreased the maximum histamine response, but did not shift the histamine dose-response to the right. Blockade by promethazine of the histamine cardiac effects could not be overcome by increasing the concentration of the agonist, which is an indication of a noncompetitive or competitive nonequilibrium type of antagonism. Only one concentration of promethazine ($4 \times 10^{-6} \text{ M}$) did not affect the chronotropic effect of histamine in the guinea pig heart (Fig. 23) whereas higher concentrations ($8-16 \times 10^{-6} \text{ M}$) of promethazine reduced the chronotropic response of histamine. The increase in promethazine concentration ($16 \times 10^{-6} \text{ M}$) further reduced the histamine effect on heart rate. The chronotropic effects of histamine are reduced by promethazine in a noncompetitive or nonequilibrium manner.

Histamine (1 μg) caused about a four fold increase in the tissue cyclic AMP content. Promethazine ($4 \times 10^{-6} \text{ M}$) reduced the histamine-induced cardiac cyclic AMP levels (Fig. 24). Increasing the dose of histamine to 3.2 μg in the presence of promethazine ($4 \times 10^{-6} \text{ M}$) did not further increase the levels of cyclic AMP. Again the type of blockade noted with promethazine was noncompetitive or competitive nonequilibrium.

The interaction between promethazine and norepinephrine was studied on guinea pig heart. The data are summarized in Table 6. Promethazine ($2-4 \times 10^{-6} \text{ M}$) did not affect the positive inotropic response to norepinephrine. At $8 \times 10^{-6} \text{ M}$, promethazine lowered the maximum response to norepinephrine. Promethazine ($16 \times 10^{-6} \text{ M}$) decreased the effects of all doses of norepinephrine.

In summary, the results of the experiments described this far suggest

FIG. 20. The effect of various concentrations of promethazine on cardiac force of contraction. Results are presented as percentage increase over control versus dose of promethazine perfused through the heart. Each point represents the mean \pm S.E. of four hearts. Force was significantly elevated at 2×10^{-6} and 4×10^{-6} M.

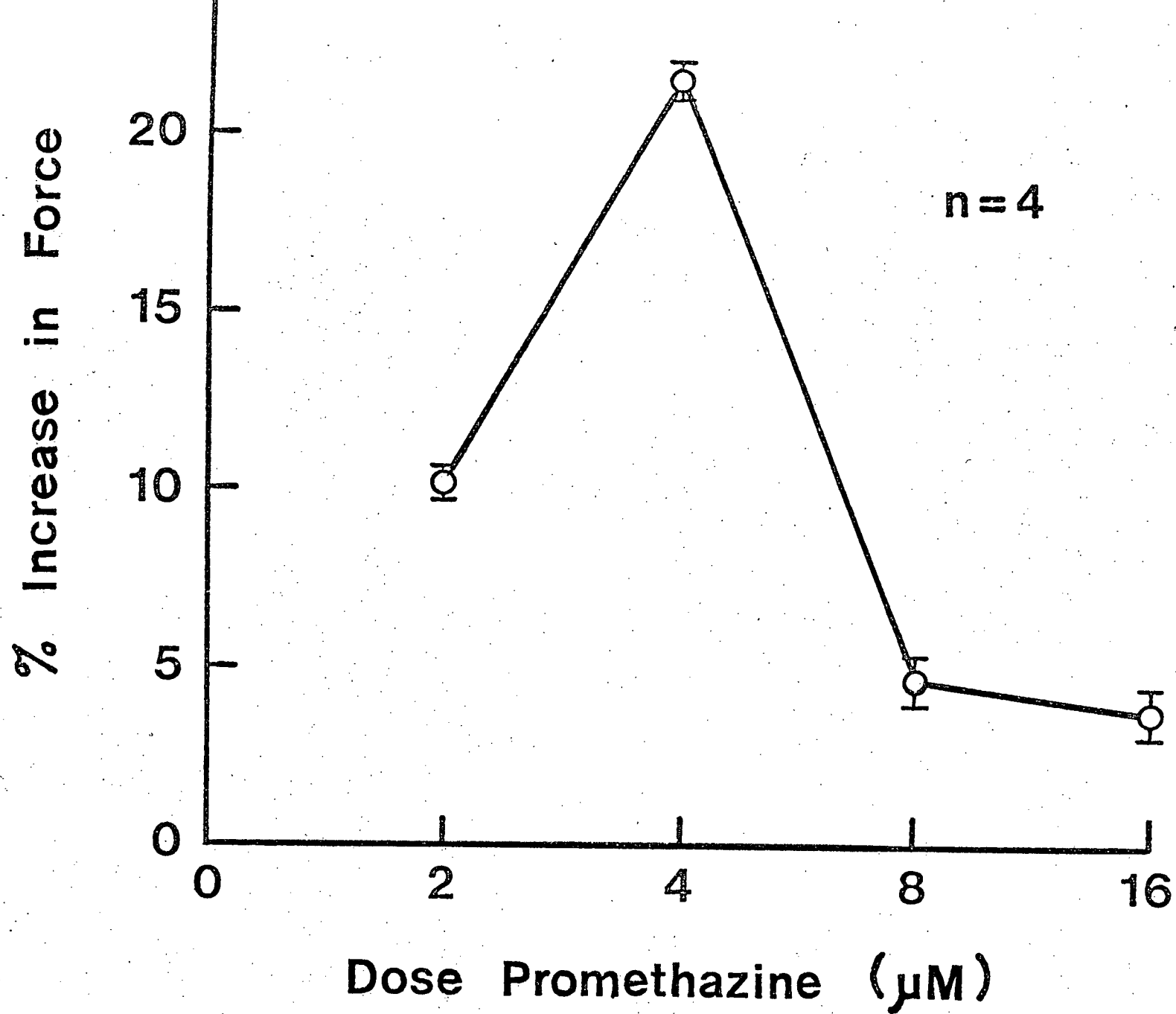


FIG. 21. The effect of various concentrations of promethazine on heart rate. Heart rate was significantly elevated at 4×10^{-6} M promethazine and significantly lowered at 8×10^{-6} and 16×10^{-6} M promethazine.

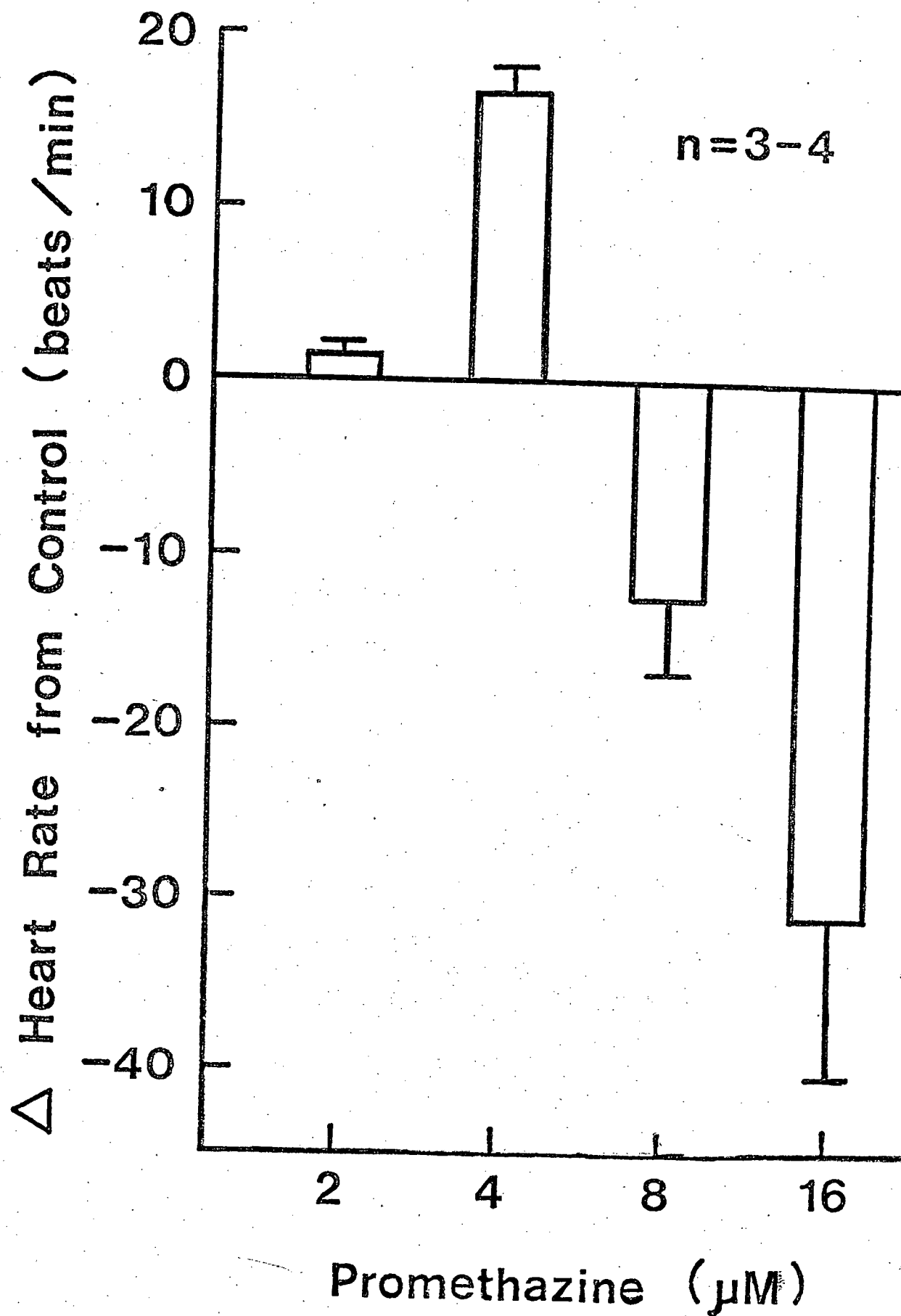
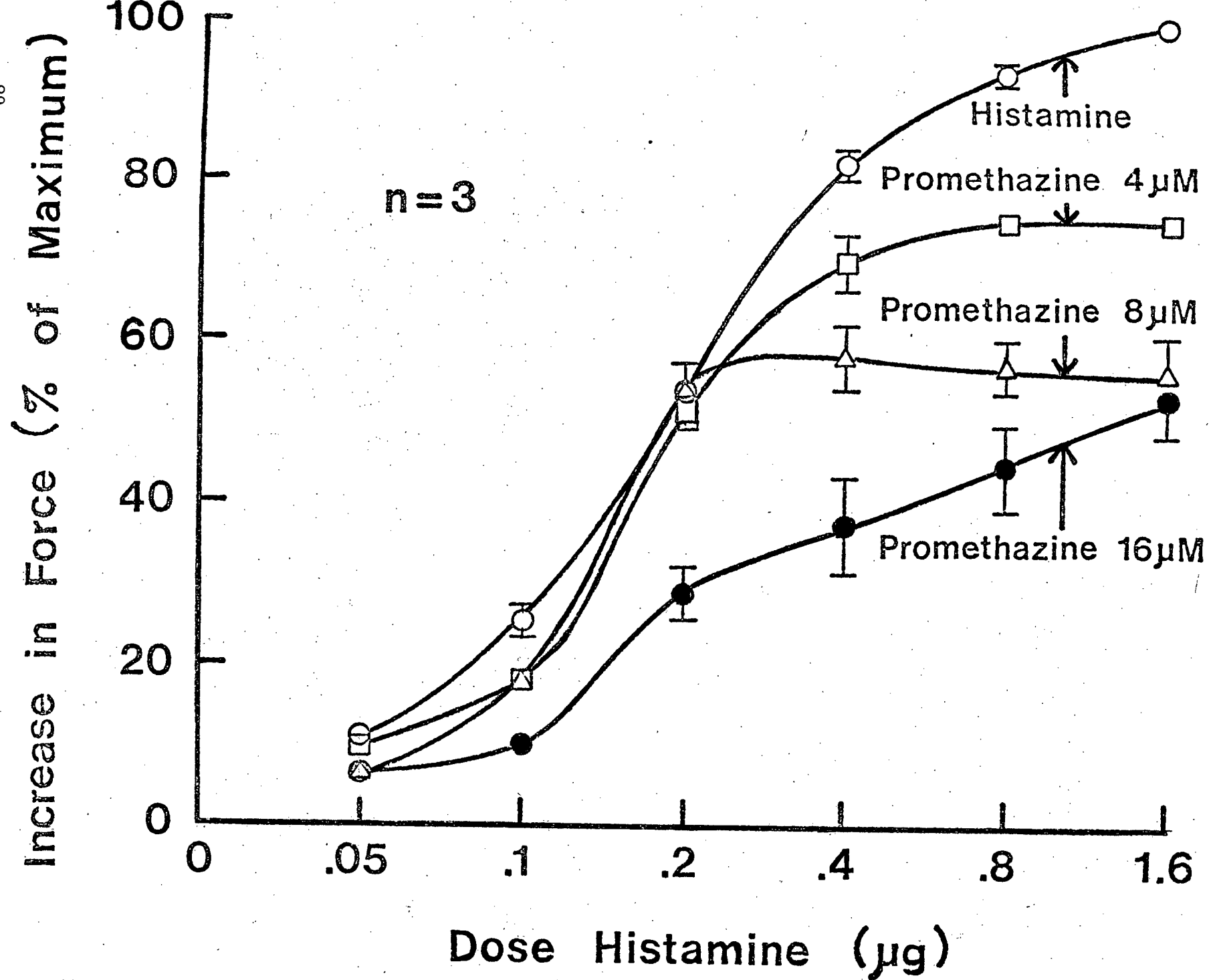


FIG. 22. The effect of promethazine (4×10^{-6} - 16×10^{-6} M) on the histamine-induced increase in force of contraction.



that burimamide, competitively blocked the histamine cardiac effects, whereas promethazine interacted with the cardiac histamine receptors, but in a non-competitive or non-equilibrium manner.

Histamine-cardiac adenylylase interaction

In order to further characterise the H_2 receptor in heart, the effects of histamine on cardiac adenylylase were studied. Fig. 25 illustrates the data obtained with several doses of histamine, 4-methylhistamine (a specific H_2 receptor agonist; (Black et al. 1972), TD and betazole. All the agonists, stimulated cardiac adenylylase. The data were plotted as agonist concentration vs cyclic AMP formed (P Mol./mg. protein/min.). The order of potency of the compounds for stimulating cardiac adenylylase was histamine>4-methylhistamine>TD>betazole. The activities of 4-methylhistamine and TD were not significantly different from each other, but were significantly greater than the activity of betazole. Burimamide ($1 \times 10^{-5} M$ and $5 \times 10^{-6} M$) shifted the histamine dose-response curve to the right. Burimamide ($10^{-6} M$) also produced a shift in the dose-response curve to the 4-methylhistamine stimulation of cardiac adenylylase (Fig. 26).

The study of the two other histamine analogs, TD and betazole, on cardiac adenylylase and their interaction with burimamide is presented in Fig. 27. Burimamide ($10^{-6} M$) moved the TD and betazole-induced stimulation of the cardiac adenylylase to the right. The burimamide blockade could be overcome by increasing the concentration of the agonists. Thus burimamide competitively blocked the histamine and histamine analog-induced stimulation of cardiac adenylylase.

FIG. 23. The effect of promethazine (4×10^{-6} - 16×10^{-6} M) on the histamine-induced increase in heart rate.

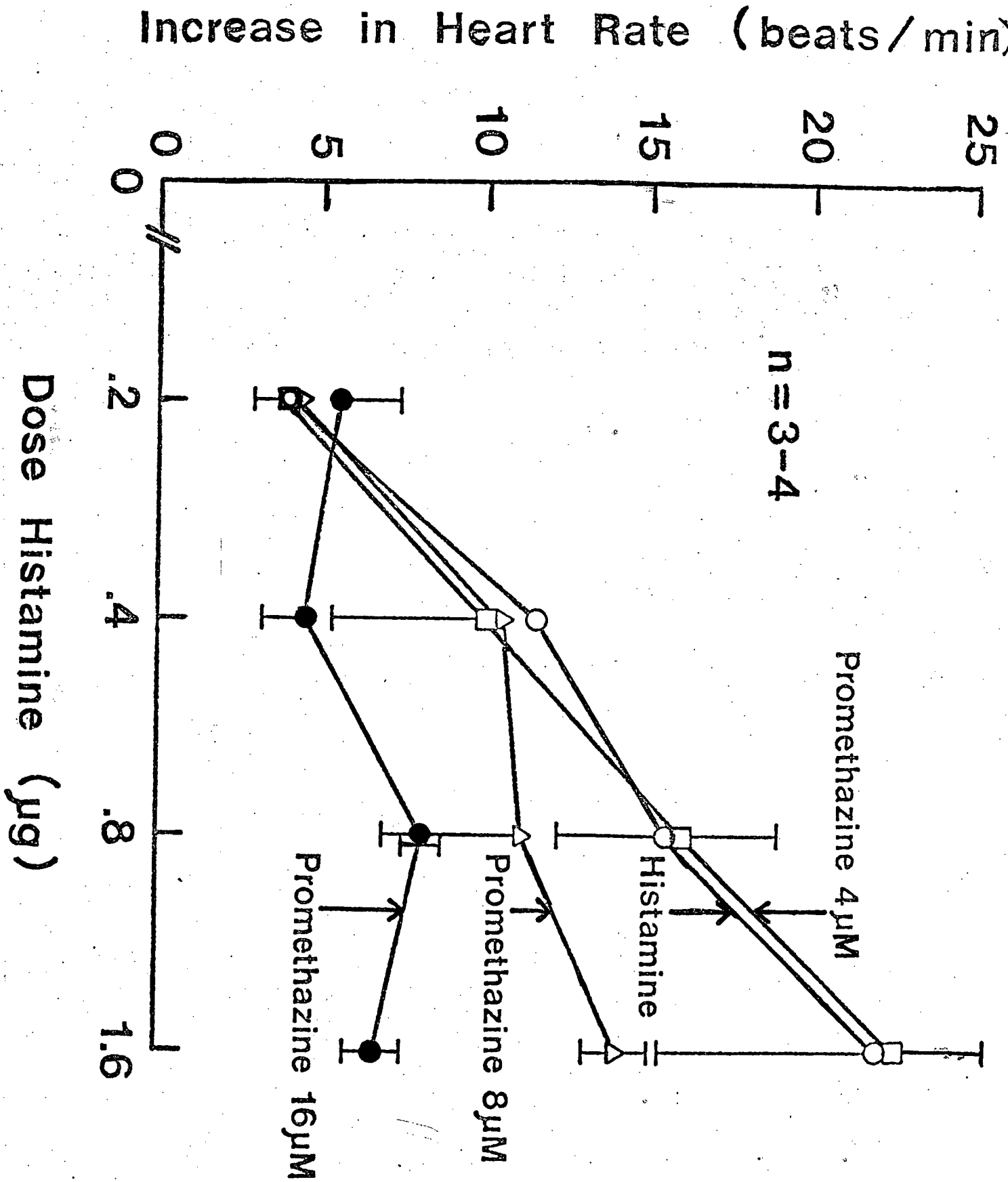


FIG. 24. The effect of promethazine (4×10^{-6} M) on the histamine-induced increase in cardiac cyclic AMP.

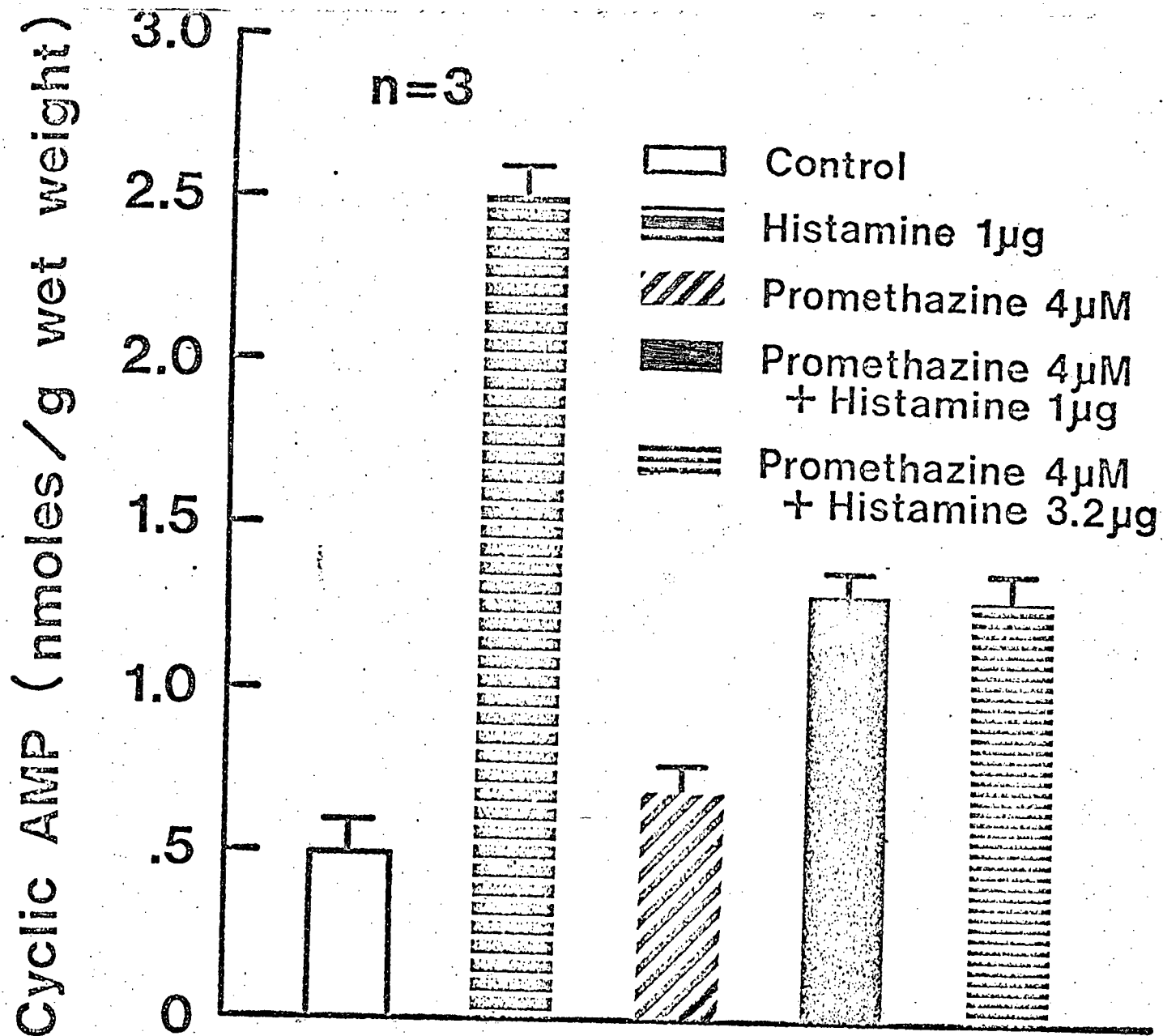


FIG. 25. The effect of various doses of histamine, 4-methylhistamine, TD and betazole on the activity of guinea pig cardiac adenylate cyclase. ○ Histamine, ◒ 4-Methylhistamine, ... TD, ◑ Betazole.

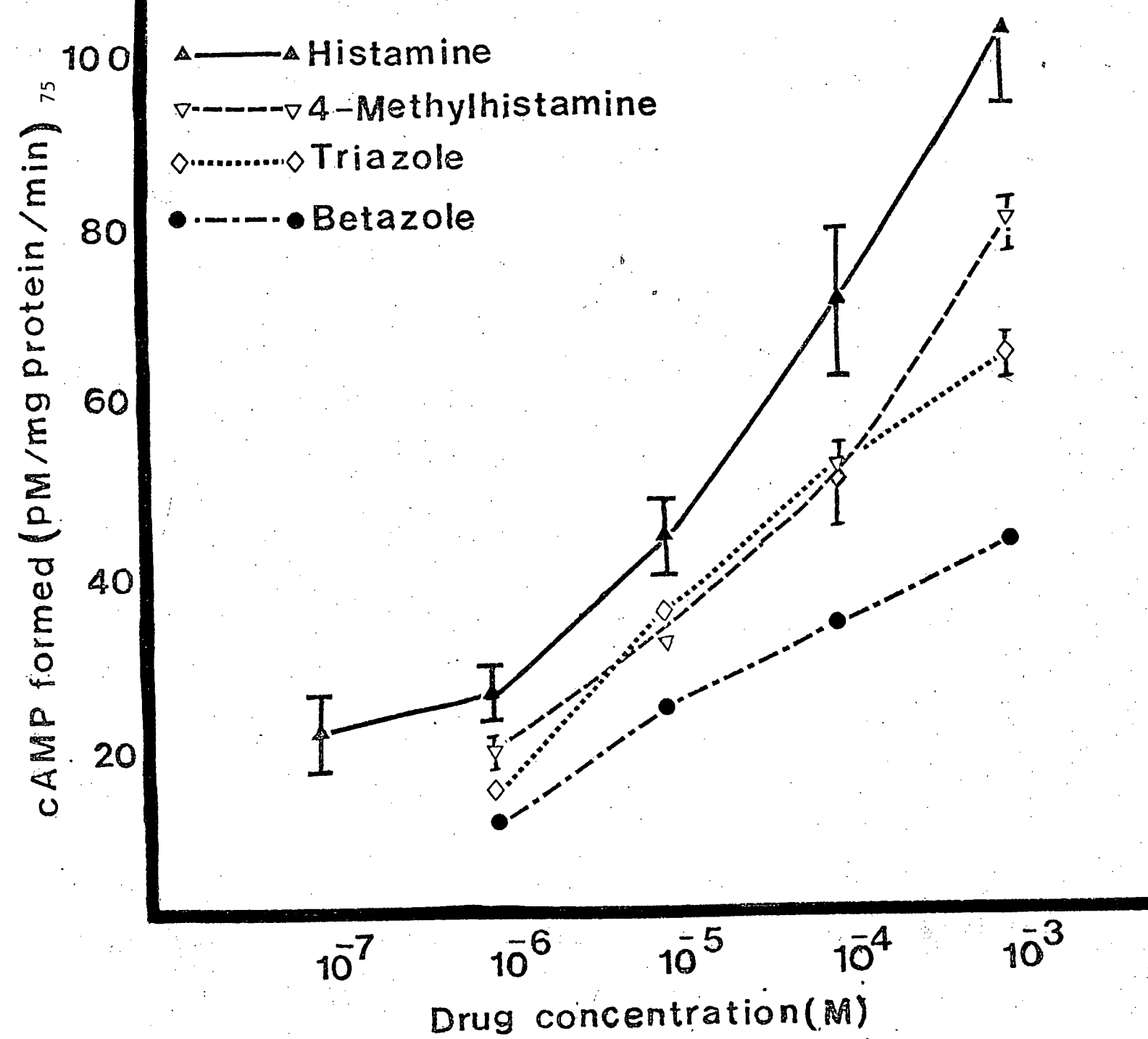


TABLE 6

The effects of promethazine, 2×10^{-6} - 16×10^{-6} M, on the norepinephrine induced increase in cardiac force.

Dose of norepinephrine	Concentration of promethazine (M)				
	0	2×10^{-6}	4×10^{-6}	8×10^{-6}	16×10^{-6}
0.2	50.2 ± 2.31^a	48.6 ± 1.21	50.7 ± 3.61	43.8 ± 1.43	38.3 ± 1.15^b
0.4	75.7 ± 1.52	73.8 ± 0.89	74.2 ± 2.12	71.2 ± 0.93	62.0 ± 1.15^b
0.8	90.5 ± 0.98	89.7 ± 1.21	89.8 ± 1.80	83.2 ± 0.87	78.3 ± 1.93^b
1.6	100	98.3 ± 1.31	98.4 ± 1.51	90.0 ± 0.46^b	86.0 ± 0.86^b

a. Means \pm S.E.M. of three hearts.

b. Significantly less than control $P < 0.01$.

NOTE: The results are given as percentage of the maximum response that could be obtained with norepinephrine.

Histamine-gastric adenylate cyclase interaction

At this stage it was of interest to determine whether the association of H_2 receptors with adenylate cyclase was confined to the heart or whether this association was found in other tissue as well. It has been suggested that histamine-induced gastric acid secretion in frog gastric mucosa is not blocked by H_1 type of histamine antagonists. Black et al. (1972) suggested histamine gastric receptors were of the H_2 type. Therefore the interaction between histamine and gastric adenylate cyclase was studied. Fig. 28 shows that histamine, 4-methylhistamine, TD and betazole all stimulated gastric adenylate cyclase. Histamine-induced stimulation of adenylate cyclase was significantly greater than 4-methylhistamine, TD and betazole. Again there was no significant difference between 4-methylhistamine and the TD. The rank order of the compounds with regard to their respective activities was the same as observed with cardiac adenylate cyclase. Fig. 29 shows that burimamide ($5 \times 10^{-6} M$) and $1 \times 10^{-6} M$) shifted the curves of histamine and 4-methylhistamine respectively to the right. The burimamide blockade was overcome by increasing the concentration of the agonists. Fig. 30 illustrates, TD and betazole stimulated gastric adenylate cyclase dose-response curves were shifted to the right by burimamide ($10^{-6} M$). The data from these experiments suggests that burimamide competitively antagonized the histamine-induced activation of gastric adenylate cyclase. Our results suggest that histamine receptors in the heart and in the stomach are associated with adenylate cyclase.

Histamine-myometrium adenylate cyclase interaction

Histamine effects on the rat uterus are not blocked by the ~~H₁~~ antihistaminic drugs (Ash and Schild 1966). Black et al. (1972) reported that burimamide,

FIG. 26. The effect of burimamide (5 to 10×10^{-6} M) on the stimulation of guinea pig cardiac adenylate cyclase by various doses of histamine and 4-methylhistamine. — Histamine, - - - Histamine + burimamide (5×10^{-6} M), . . . Histamine + burimamide (1×10^{-5} M). 4-Methylhistamine, - - - 4-Methylhistamine + burimamide (1×10^{-6} M).

cAMP formed (pM/mg Protein/min)

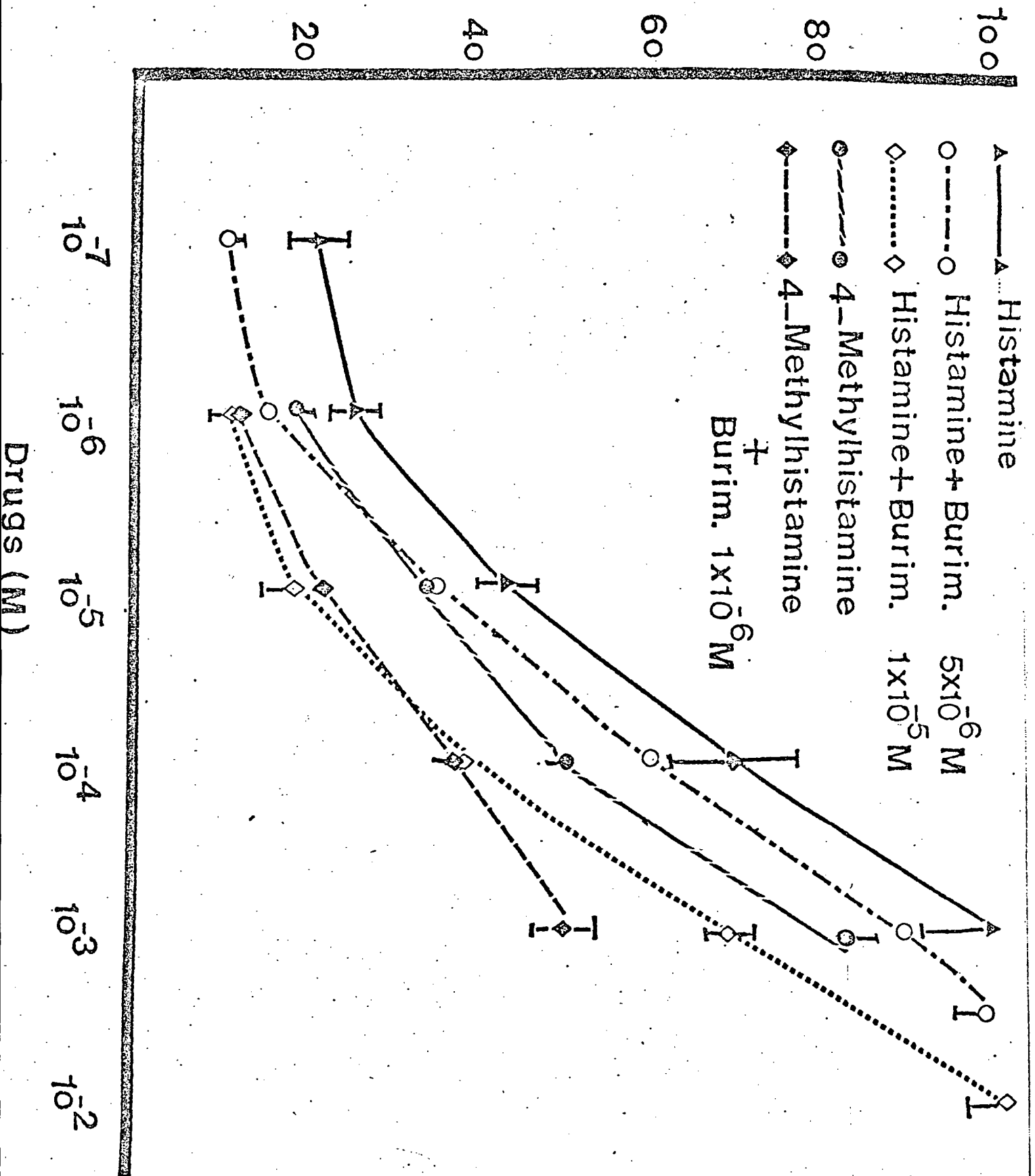


FIG. 27. The effect of burimamide ($1 \times 10^{-6} \text{M}$) on the stimulation of guinea pig cardiac adenylate cyclase by various doses of betazole and TD. Each point represents the mean \pm S.E. of 4 determinations. TD, Betazole, --- TD + burimamide ($1 \times 10^{-6} \text{M}$), --- Betazole + burimamide ($1 \times 10^{-6} \text{M}$).

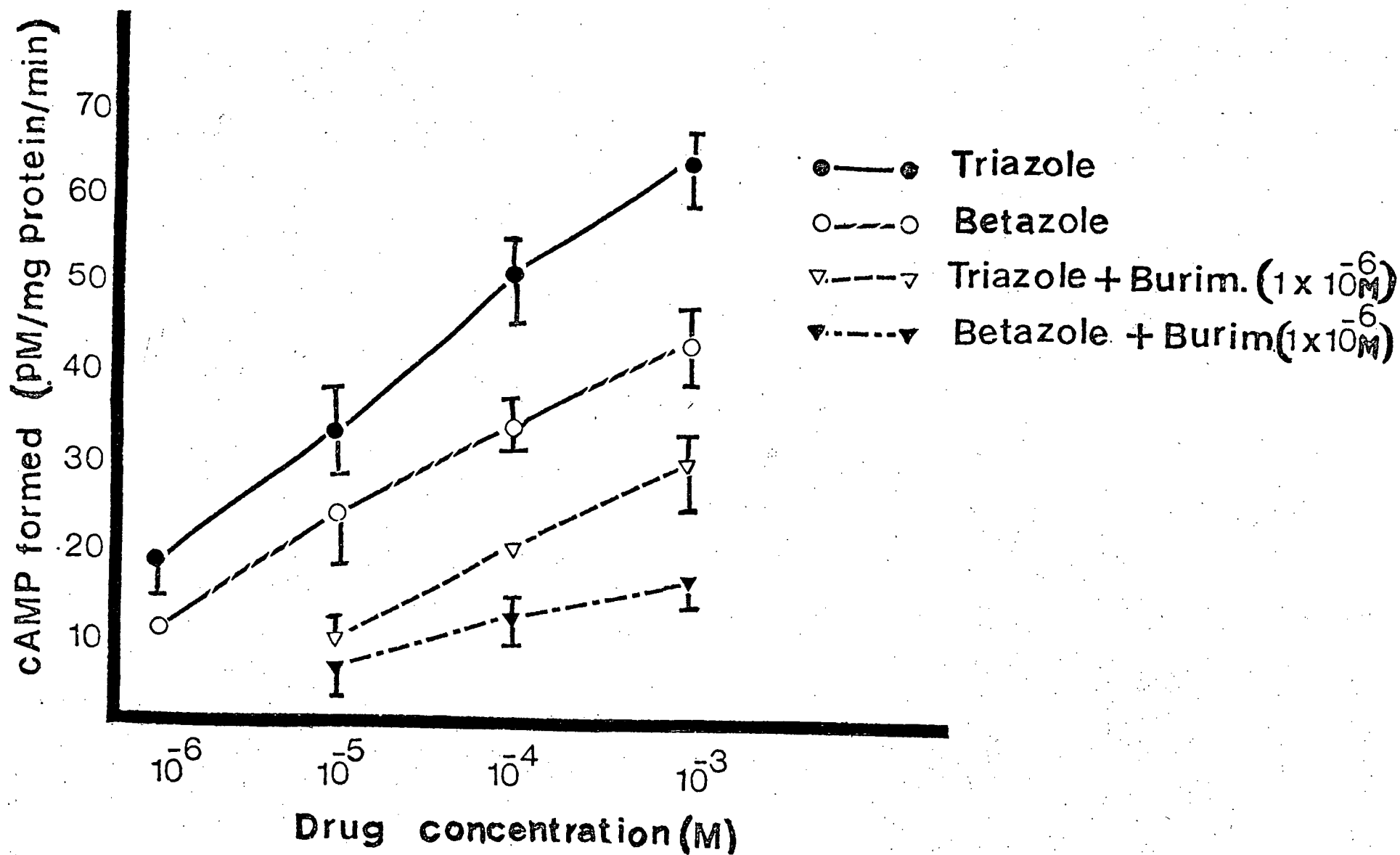


FIG. 28. The effect of various concentrations of histamine, 3-(β -aminoethyl)-1,2,4 triazole (Triazole), 4-methylhistamine and betazole on rat gastric adenylate cyclase activity. Each point represents the mean \pm S.E.M. of 4 determinations.

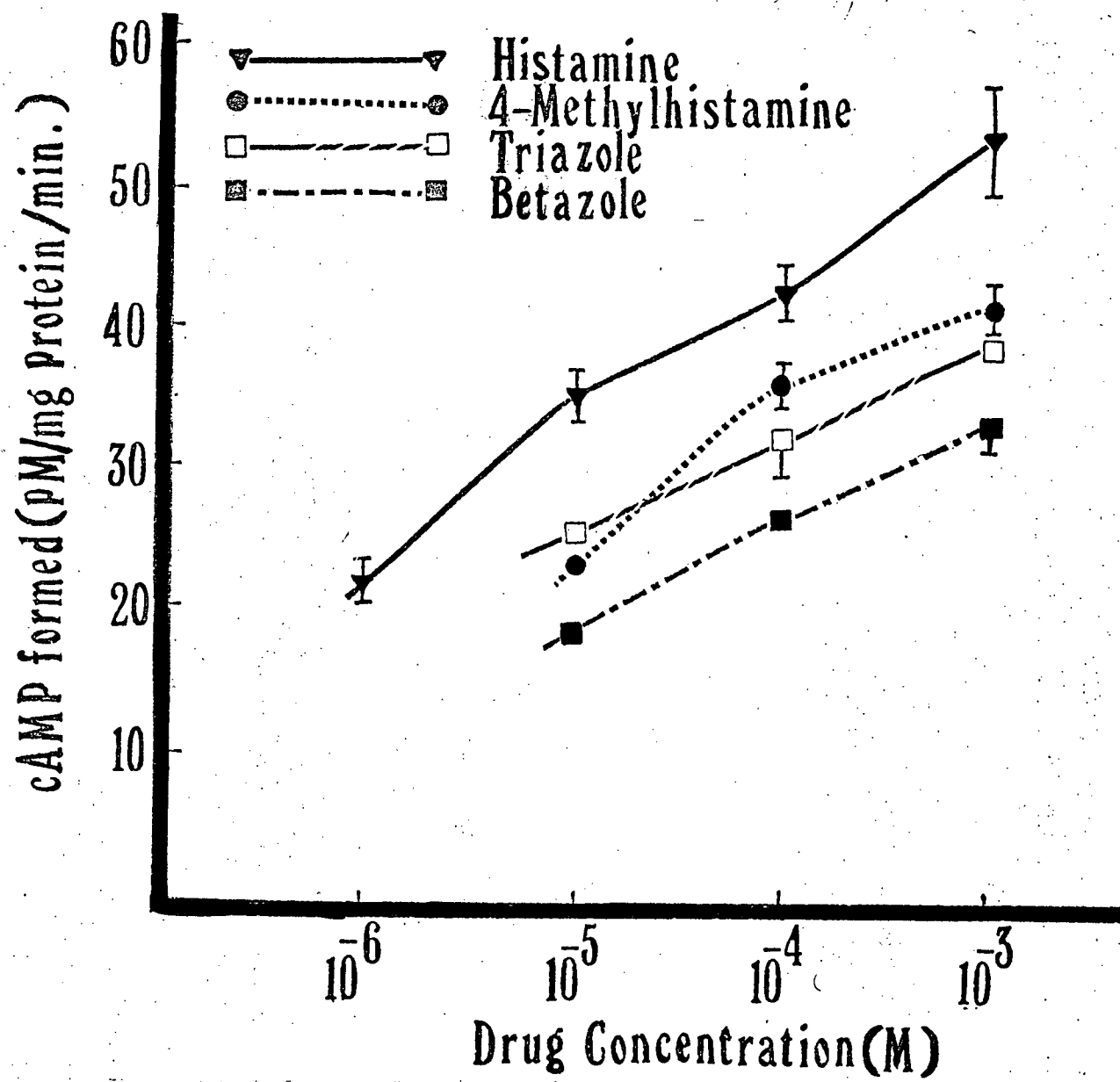


FIG. 29. The effect of various concentrations of histamine and 4-methylhistamine and the interaction of these drugs with burimamide on rat gastric adenylate cyclase activity. Each point represents the mean \pm S.E.M. of 4-6 determinations.

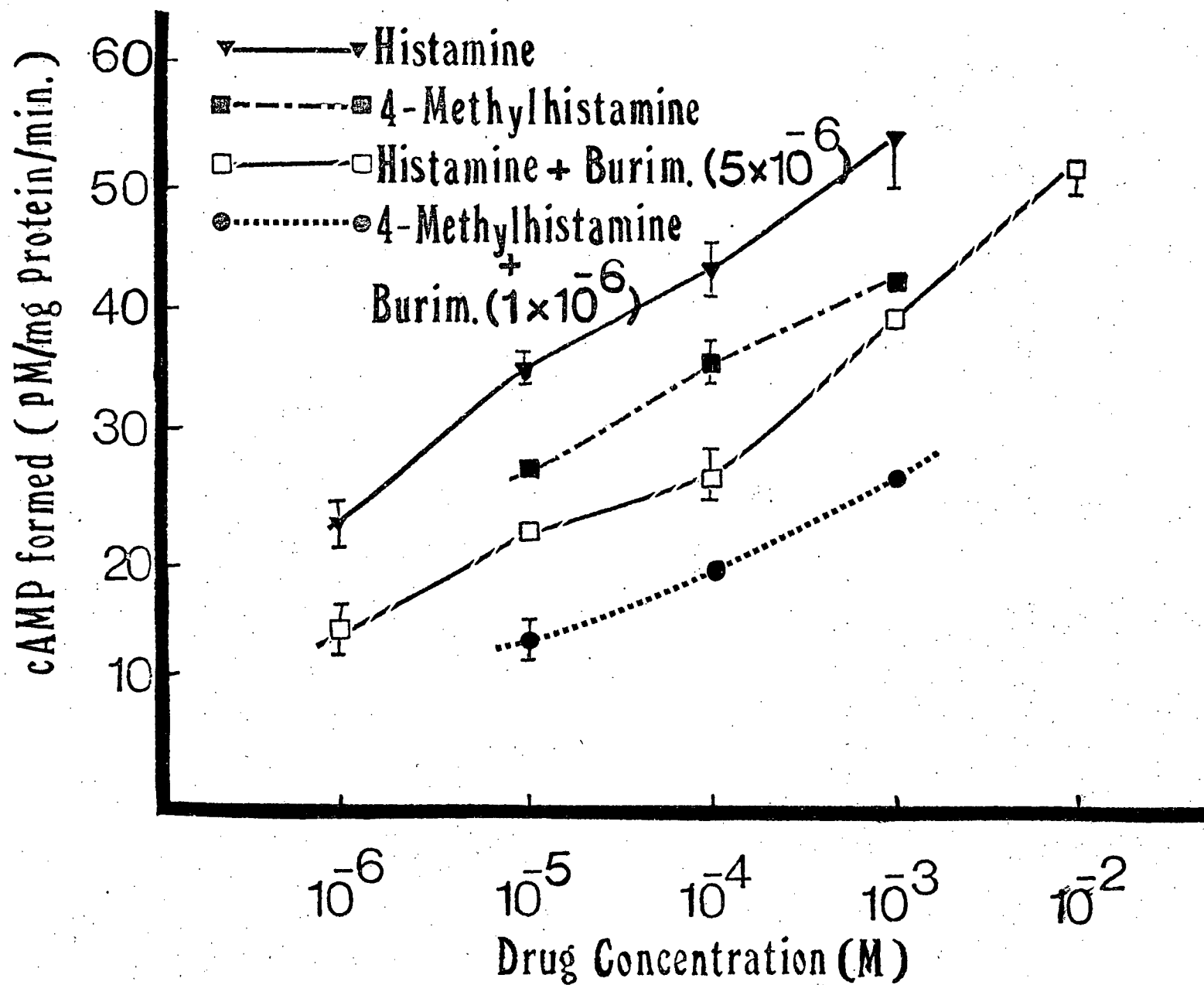
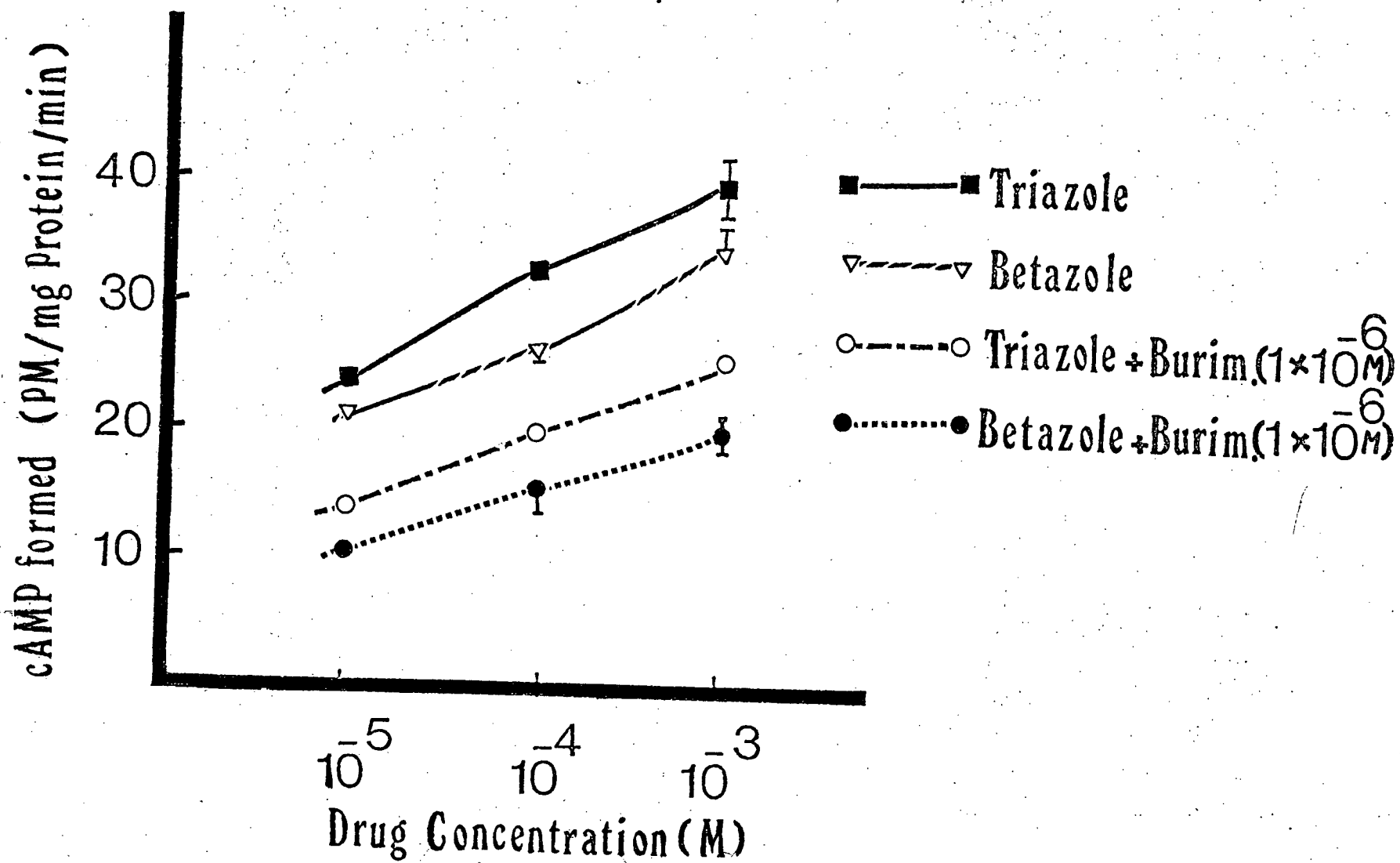


FIG. 30. The effect of 3-(β -aminoethyl)-1,2,4 triazole (Triazole) and betazole and the interaction of these drugs with burimamide on rat gastric adenylate cyclase activity. Each point represents the mean \pm S.E.M. of 4 determinations.



an H_2 -receptor antagonist blocked the histamine effects on rat uterus. A dose-response study of the effects of histamine and its analogs on the rat uterus is shown in Fig. 31. It demonstrates that histamine, 4-methylhistamine, TD and betazole all relaxed the rat uterus in a dose-dependent manner. The order of potency was histamine > 4-methylhistamine > TD > betazole. A study of the interaction of histamine on the adenylate cyclase particles prepared from rat uterus, revealed that histamine did not stimulate the adenylate cyclase.

Histamine-guinea pig ileum interaction

Preliminary experiments established that 10^{-5} M histamine produced a maximum contraction of the guinea pig ileum. Burimamide did not block the histamine-induced contractions in the guinea pig ileum, whereas diphenhydramine, a H_1 receptor antagonist competitively blocked the histamine effects on guinea pig ileum (Ash and Schild 1966). Stimulation of cardiac H_2 -receptors in either guinea pig heart or rat stomach resulted in the activation of adenylate cyclase and subsequent formation of cyclic AMP (present study).

In a time-response study histamine (10^{-5} M) did not increase cyclic AMP at any time tested (Table 7) or table 7.

Cardiac actions and interaction of theophylline with norepinephrine and histamine

The inotropic and phosphorylase activating effects of adrenergic and histaminergic drugs have been suggested to be mediated through cyclic AMP. Theophylline, a phosphodiesterase inhibitor potentiates the amine effects on

FIG. 31. The effect of various concentrations of histamine, 4-methylhistamine, 3-(β -aminoethyl)-1,2,4 triazole (Triazole) and betazole on the rat uterus. Each point represents the mean \pm S.E.M. of 3 determinations.

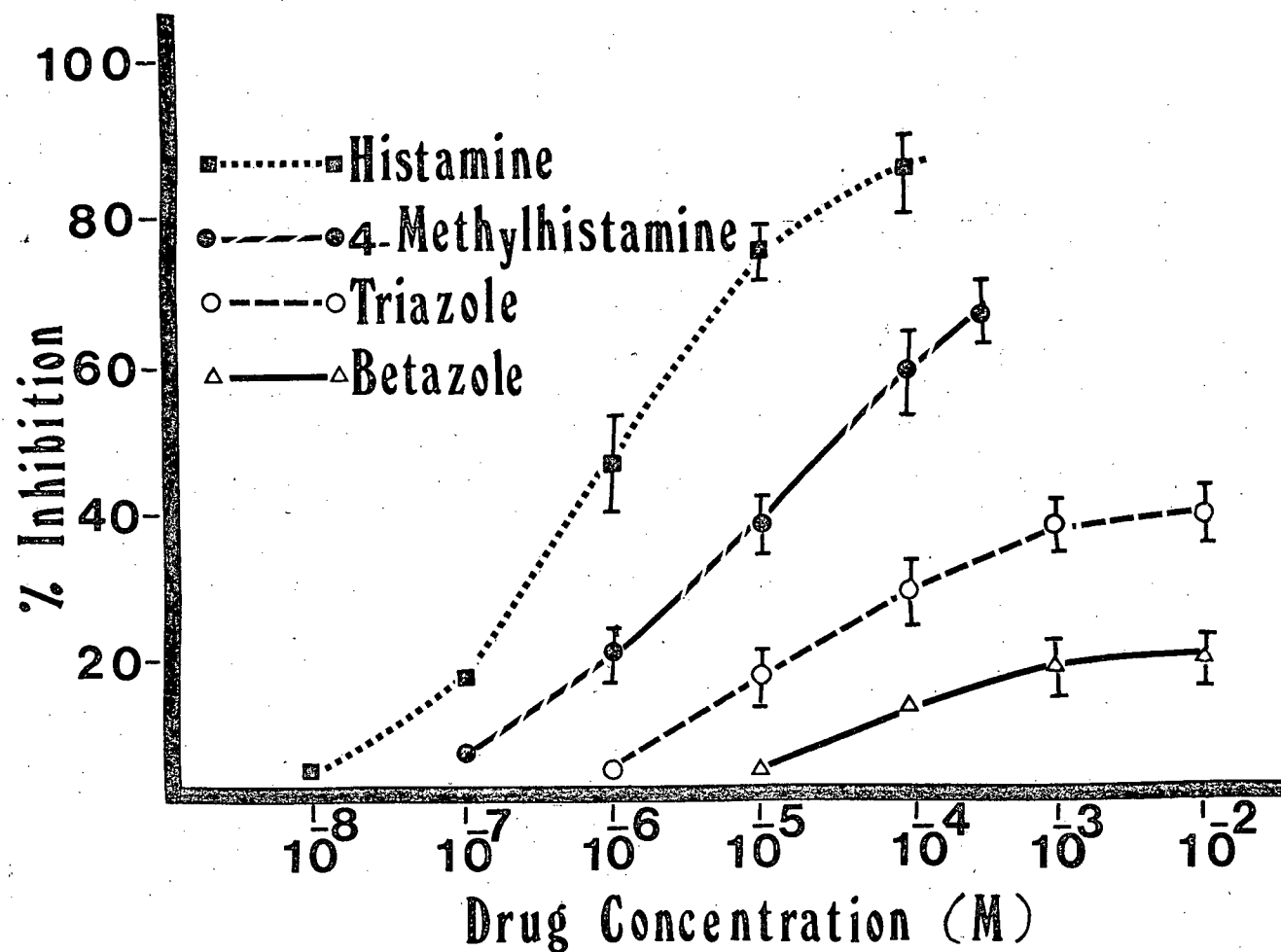


TABLE 7

The effect of histamine (10^{-5} M) at various times on the level of cyclic AMP in the guinea pig ileum. Each result is the mean of 5-6 determinations \pm S.E.

Time (Sec)	Cyclic AMP (nmol/gm wet weight)
0	0.58 \pm 0.088
5	0.66 \pm 0.080
10	0.56 \pm 0.070
20	0.50 \pm 0.050
30	0.63 \pm 0.051
50	0.64 \pm 0.030

the heart and is also capable of directly stimulating the heart. Therefore the effects of theophylline on cardiac contractility, cyclic AMP and phosphorylase activation as well as the interaction between norepinephrine or histamine and theophylline on these parameters were investigated.

Theophylline (1.0 mg) when injected into the perfused guinea pig heart caused a 20.0% increase in cardiac contractility over the control (Fig. 32). In about 20-30 seconds slight phosphorylase a activation was noted. At no time were significant differences in cyclic AMP levels observed. Control phosphorylase a levels were $2.9 \pm 0.3\%$, which increased to $6.5 \pm 1.2\%$ at about 20 sec. and remained elevated over the 50 sec. experimental period. Control levels of cyclic AMP were 0.54 ± 0.07 nmoles/gm wet wt. and did not change over the experimental time interval.

In the next set of experiments, the interaction between theophylline and norepinephrine or histamine were investigated. Theophylline, at a concentration of $7.0 \times 10^{-4} \text{M}$, was perfused for 15 min. through the isolated rat heart. This was the minimal concentration of theophylline necessary to enhance the inotropic effects of norepinephrine. Theophylline significantly potentiated the 0.2 and 0.4 μg doses of norepinephrine effects on phosphorylase a activation (Fig. 33).

As stated before, theophylline ($7 \times 10^{-4} \text{M}$) produced an increase in cardiac contractility. However an increase in the dose of theophylline to $2 \times 10^{-3} \text{M}$, resulted in a negative inotropic effect. When the hearts were perfused with theophylline ($2 \times 10^{-3} \text{M}$) and injected with different doses of norepinephrine, the norepinephrine response was lowered (Fig. 34). Theophylline ($7 \times 10^{-4} \text{M}$ or $2 \times 10^{-3} \text{M}$) did not potentiate the norepinephrine-induced elevation of cyclic AMP (Fig. 34).

Similar experiments were carried out in the guinea pig hearts, perfused

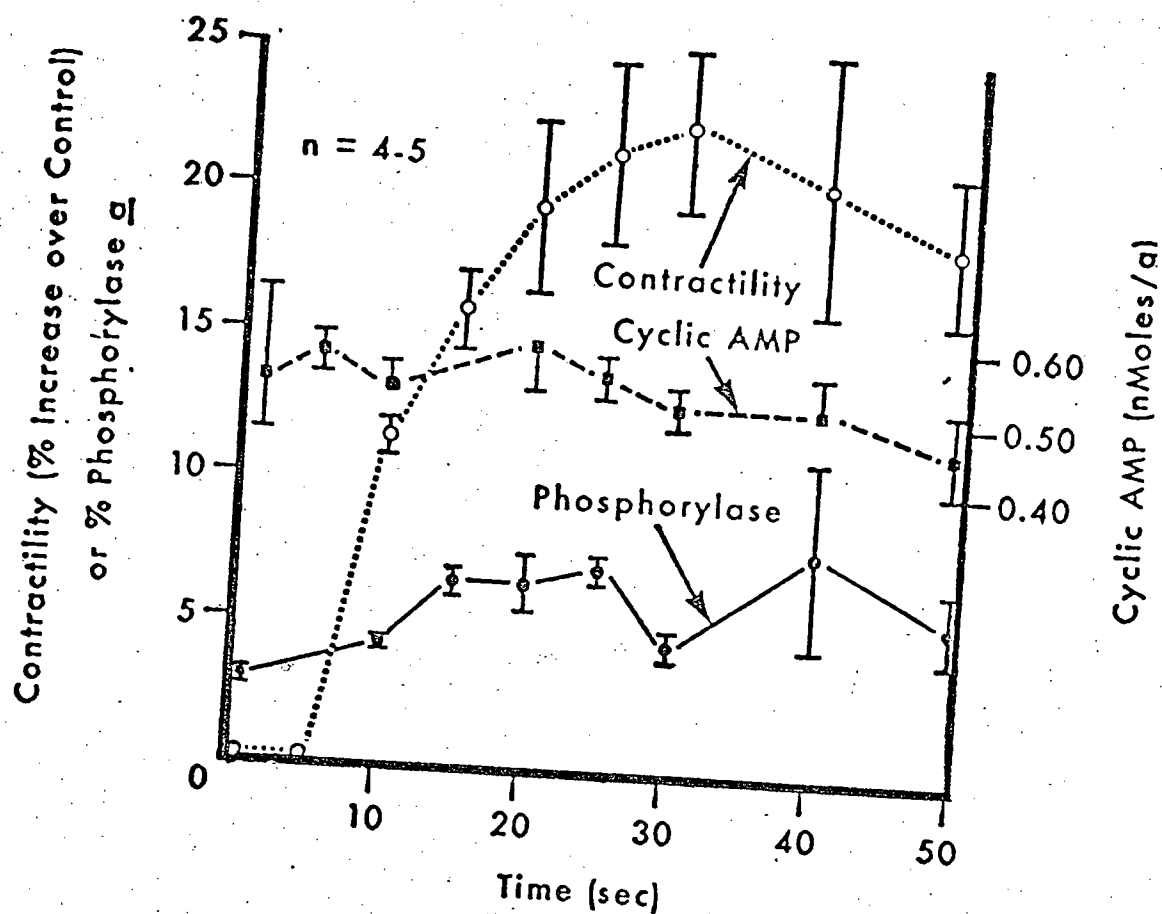


FIG. 32. The effect of theophylline (1 mg) at various times following injection into the perfused guinea pig heart on contractility, % phosphorylase a and cyclic AMP.

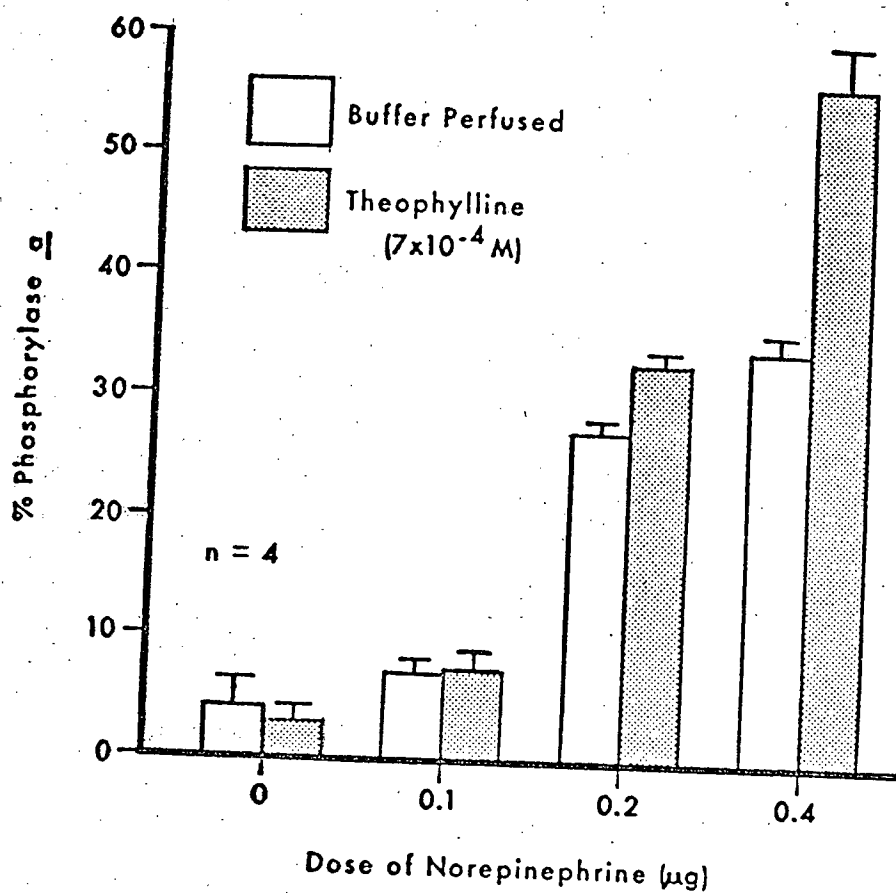


FIG. 33. The effect of various doses of norepinephrine on cardiac phosphorylase activation in rat hearts perfused with buffer or buffer plus theophylline ($7 \times 10^{-4} M$).

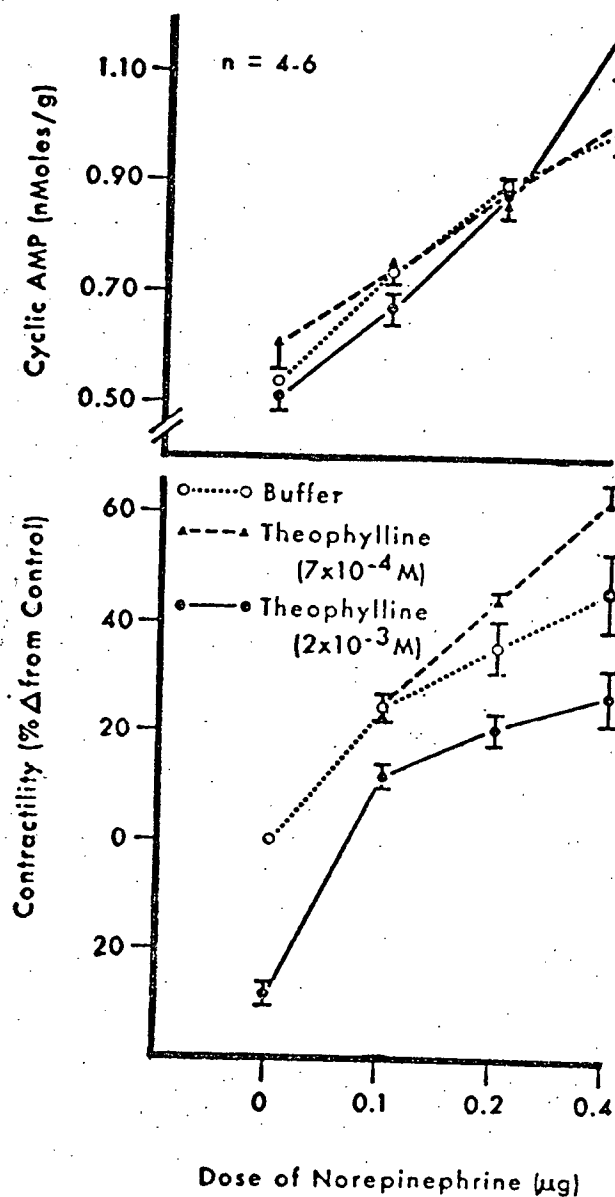


FIG. 34. The effect of various doses of norepinephrine on cardiac contractility and cyclic AMP in rat hearts perfused with buffer or buffer plus theophylline ($7 \times 10^{-4} \text{M}$ or $2 \times 10^{-3} \text{M}$).

with buffer or buffer plus 10^{-3} M theophylline and injected with either buffer or with different doses of histamine. In these experiments, theophylline (10^{-3} M) potentiated the histamine (0.2 and 0.4 μ g) cardiac effects on contractility and phosphorylase a (Fig. 35).

Histamine (0.2 and 0.4 μ g) induced increases in cyclic AMP were not further increased by the presence of theophylline (Fig. 35).

Cardiac actions and interaction of imidazole with norepinephrine and histamine

The cardiac actions of imidazole, a phosphodiesterase stimulator, and the effects of this drug with norepinephrine and histamine were investigated. Imidazole perfusion in the concentration range of 0.25 to 40mM resulted in a concentration related increase in cardiac contractility (Fig. 36). In a similar set of experiments different doses of imidazole (0.05-1.6 mg) were injected by a side arm cannula into the perfused guinea pig heart. Imidazole produced a dose-dependent increase in cardiac contractility (Fig. 37). The maximum increase in force with 40mM imidazole perfusion was $31.3 \pm 6.3\%$. A dose of 1.6 mg of imidazole produced a $23.7 \pm 3.3\%$ increase in contractility over the control levels.

Further experiments were carried out to test the interaction between imidazole and histamine or norepinephrine, in the guinea pig hearts perfused with 40mM imidazole. Data in table 8 show that imidazole (40mM) reduced the histamine and norepinephrine dose-response effects on cardiac contractility. Results in Table 8, are expressed as a percentage of maximum response obtained with the agonists. Similar effects with imidazole on amine-induced contractility were reported by Pösch and Kukovetz (1967). As cyclic AMP is believed to be important for the amine-induced increases in contractile force,

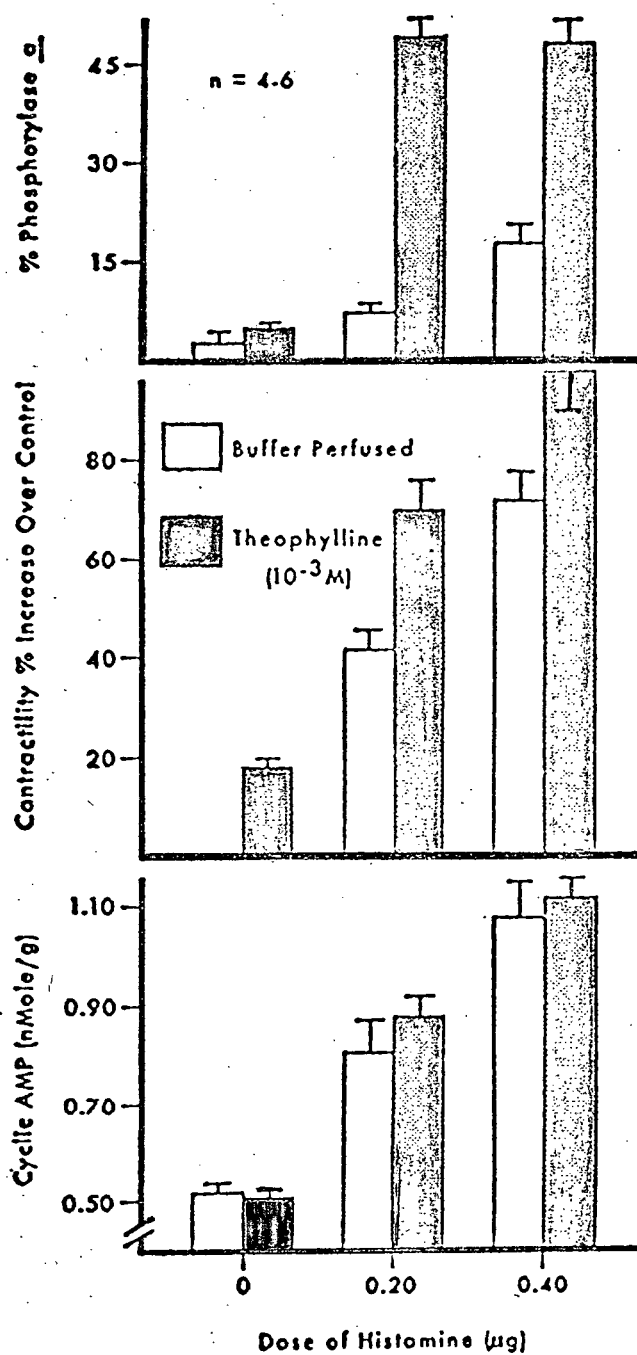


FIG. 35. The effect of various doses of histamine on cyclic AMP, contractility, and phosphorylase a in guinea pig hearts perfused with buffer or buffer plus theophylline (10^{-3}M).

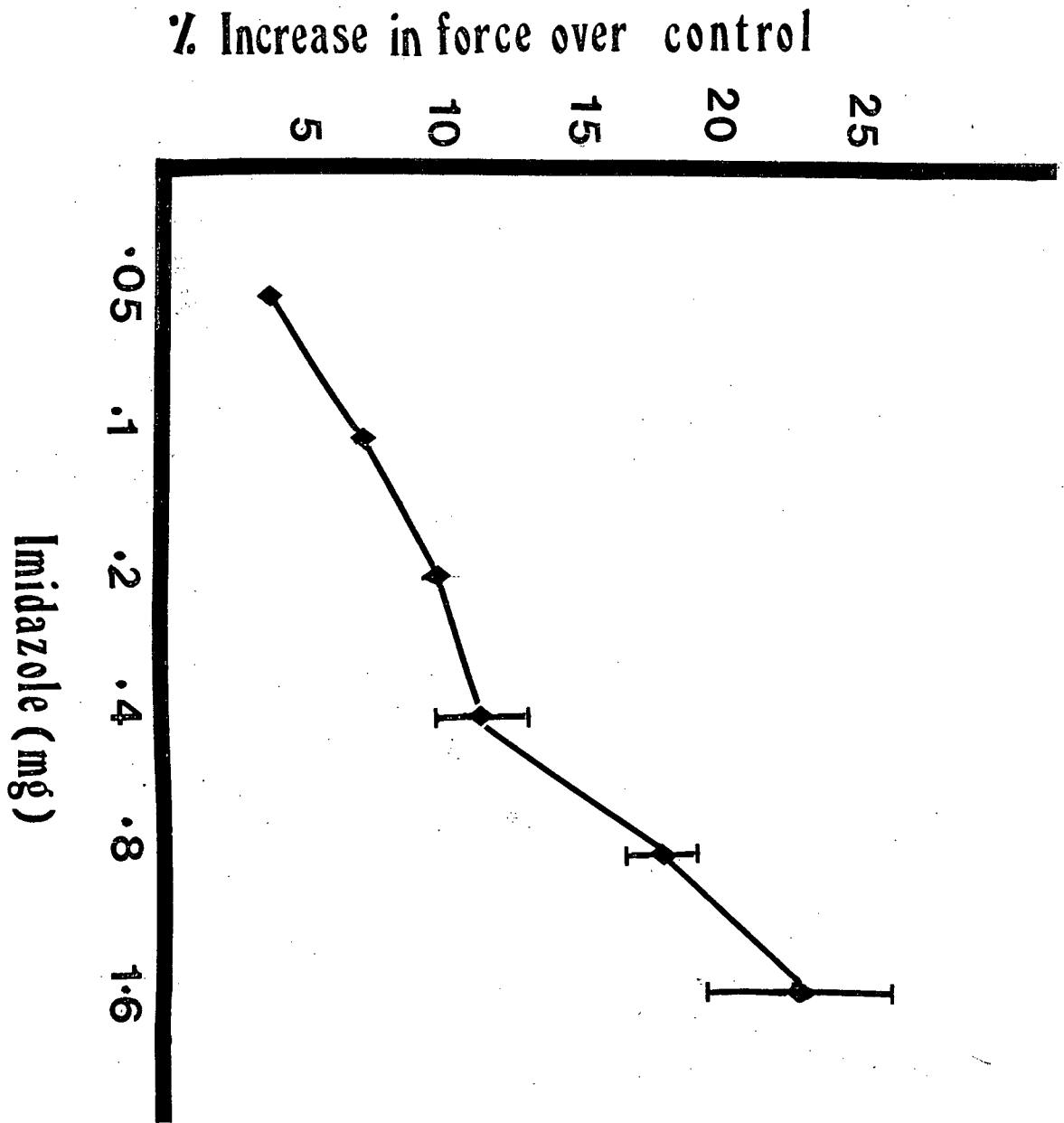


FIG. 36. The effect of various doses of imidazole on cardiac contractility in guinea pig hearts perfused with buffer. Each point represents the mean \pm S.E.M. of three determinations.

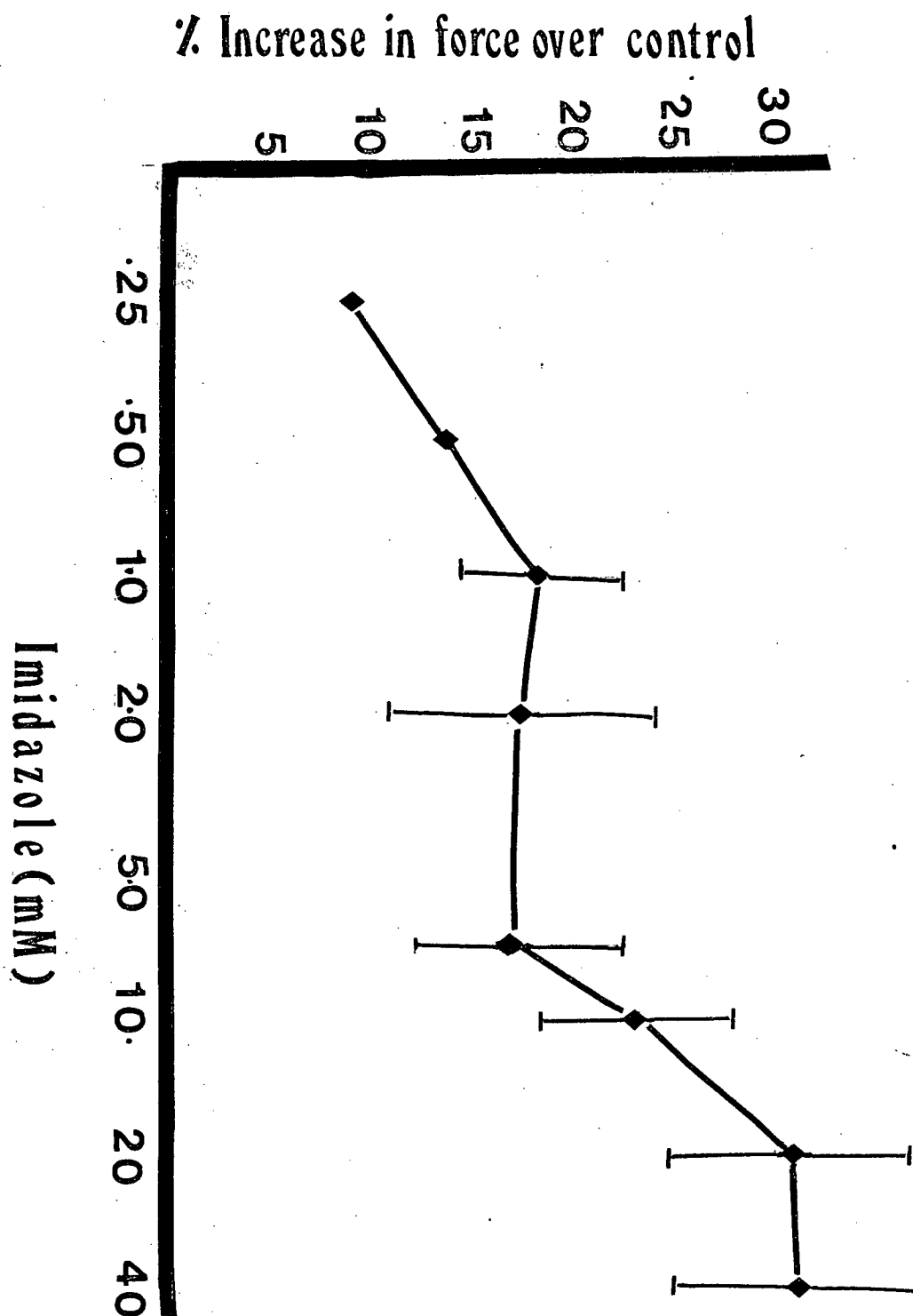


FIG. 37. The effect of perfusion of various concentrations of imidazole (0.25-40mM) in the isolated perfused guinea pig heart. Each point represents the mean \pm S.E.M. of three determinations.

TABLE 8

The effect of 40mM imidazole perfusion on the positive inotropic effect of histamine and norepinephrine.

Dose of amine (μ g)	Histamine		Norepinephrine	
	Buffer perfused	Imidazole perfused	Buffer perfused	Imidazole perfused
0.2	50.0 \pm 4.0	0.0 \pm 0.0 ^a	61.8 \pm 2.4	7.2 \pm 0.6 ^a
0.4	75.0 \pm 3.8	37.6 \pm 2.0 ^a	75.2 \pm 2.5	55.6 \pm 6.0 ^a
0.8	88.0 \pm 3.2	50.0 \pm 2.2 ^a	90.0 \pm 2.5	74.3 \pm 3.7 ^a
1.6	100.0 \pm 0.0	65.0 \pm 1.4 ^a	100.0 \pm 0.0	77.3 \pm 1.4 ^a

n = 3-4

Results are expressed as % of the maximum response that could be obtained with the agonist.

a. Significantly less than buffer perfused (P < 0.05).

TABLE 9

The effect of imidazole (40mM) on the norepinephrine and histamine induced increase in cardiac cyclic AMP.

Dose of amine (μ g)	Norepinephrine		Histamine	
	Buffer perfused	Imidazole perfused	Buffer perfused	Imidazole perfused
Control	0.45 \pm 0.017	0.44 \pm 0.018	0.45 \pm 0.017	0.44 \pm 0.018
0.25	0.75 \pm 0.045	0.52 \pm 0.031 ^a	0.70 \pm 0.05	0.48 \pm 0.018 ^a
0.5	1.12 \pm 0.050	0.91 \pm 0.037 ^a	1.05 \pm 0.075	0.67 \pm 0.036 ^a
1.0	2.25 \pm 0.100	1.22 \pm 0.040 ^a	2.30 \pm 0.022	0.78 \pm 0.058 ^a

Mean cyclic AMP (nmoles/g wet weight) of 5-7 hearts \pm S.E.

a. Significantly less than no treatment (P < 0.05).

the interaction of imidazole and histamine or norepinephrine on cardiac cyclic AMP was investigated. Guinea pig hearts were perfused with buffer or imidazole (40mM) for 15 min. and injected with various doses of histamine or norepinephrine. Imidazole perfusion decreased the amine-induced increases in cyclic AMP at all the doses tested (Table 9). Thus the decrease in cyclic AMP correlates well with the amine effects on cardiac force. In order to test the interaction of the histamine or norepinephrine on phosphorylase a activation with imidazole (40mM), the hearts were perfused with buffer or imidazole for 15 min. and injected with various doses of histamine or norepinephrine. Table 10 shows the lack of interaction between imidazole and the two amine agonists on cardiac phosphorylase a. Imidazole did not affect phosphorylase activation of the amines at any dose tested.

An investigation of the imidazole effects on cardiac cyclic AMP or phosphorylase a revealed that a 1.6 mg, imidazole injected into the perfused guinea pig heart, did produce an increase in contractility, about $23.7 \pm 3.3\%$ over control and $11.7 \pm 1.6\%$ increase in phosphorylase (Table 11). The data suggest that imidazole can produce significant changes in contractility and phosphorylase activation without measurable changes in cyclic AMP.

TABLE 10

The effect of imidazole (40mM) on the norepinephrine and histamine-induced activation of cardiac phosphorylase.

Dose of amine (μ g)	Norepinephrine		Histamine	
	Buffer perfused	Imidazole perfused	Buffer perfused	Imidazole perfused
0.0	4.8 \pm 0.87 ^a	6.6 \pm 1.00	4.8 \pm 0.87	5.5 \pm 1.00
0.25	20.6 \pm 1.87	17.0 \pm 0.57	10.3 \pm 0.63	9.0 \pm 0.76
0.5	42.0 \pm 0.57	40.0 \pm 0.57	26.9 \pm 1.10	24.3 \pm 0.33
1.0	57.4 \pm 0.70	54.6 \pm 1.70	42.4 \pm 0.69	40.2 \pm 1.47

- a. Mean % of phosphorylase a of 5-7 hearts \pm S.E. measured at the peak of the phosphorylase response.

TABLE 11

The effect of imidazole on phosphorylase a, cyclic AMP and contractile force in the isolated perfused guinea pig heart.

Dose of Imidazole	%phosphorylase <u>a</u>	cyclic AMP (nmoles/gm wet weight)	% increase in force over control
Control	4.5 ± 1.5	0.45 ± .017	—
1.6 mg	11.7 ± 1.6 ^a	0.46 ± 0.04	23.7 ± 3.3 ^a

n = 3-4

Cyclic AMP was measured in hearts frozen at 18 sec. and phosphorylase in hearts frozen at 38-40 sec. following the injection of imidazole.

a. Significantly greater than no treatment ($P < 0.05$).

DISCUSSION

The positive inotropic effect of the catecholamines has been postulated to result from an increase in the intracellular level of cyclic AMP produced by the activation of adenylate cyclase (Sutherland et al. 1966). This hypothesis was questioned by Benfey (1971) who found that phenylephrine, an adrenergic amine produced increased contractile force without producing any metabolic effects. In the present study dose-response and time-response data revealed that phenylephrine produced an increase in cyclic AMP, cardiac contractility and phosphorylase activation. These data are in agreement with the findings of ~~McNeill et al. (1972)~~ who measured phosphorylase and contractility following phenylephrine injection and suggest that phenylephrine is a weak beta-adrenergic agonist. Phenylephrine in comparison to norepinephrine has less potency and efficacy in elevating cyclic AMP, producing a positive inotropic effect or raising the levels of phosphorylase a. Phenylephrine can produce these effects in a dose-dependent manner. In the time-response study, norepinephrine produced an increase in cyclic AMP which preceded the increase in contractility and phosphorylase activation. Phosphorylase a and contractility began to increase at about the same time. Cyclic AMP reached a peak before the peak in cardiac contractility or phosphorylase a. Similar results were obtained by Robison et al. (1965) in a time-response study with epinephrine in the isolated perfused rat heart. Williamson (1966) stated that cyclic AMP and contractility reached a peak at about the same time (12 sec.) following the injection of epinephrine into the perfused rat heart.

In the time-response study with phenylephrine, phosphorylase a activation appeared to lag behind the increase in cardiac contractility. Similar results were obtained when the histamine analogs, TD and betazole were used in the

present study. The apparent lag in phosphorylase may merely reflect a technical inability to detect the smaller changes in phosphorylase activation that occurred with less potent agonists. Benfey (1971) did not find an increase in cyclic AMP with phenylephrine. Benfey and Carolin (1971) have also shown that phenylephrine does not activate adenylate cyclase. Benfey and Carolin (1971) have interpreted their data to mean that phenylephrine did not stimulate adenylate cyclase and subsequently caused no elevation in cyclic AMP levels. The possible reasons for the discrepancies between the results of Benfey and those found in the present investigation are many. 1.) Benfey estimated the cyclic AMP levels in rabbit heart slices. 2.) It is very likely that the fragile nature of the enzyme (adenylate cyclase) which is easily denatured by usual laboratory physical forces such as washing, freeze thawing, homogenization and sonication, presents serious pitfalls in the interpretation of data. 3.) Benfey (1971) did not use a beating heart in which physiological parameters could be simultaneously monitored. McNeill et al. (1972) stated that phenylephrine did not possess sufficient intrinsic activity to activate the enzyme. Our data suggests that phenylephrine, like other adrenergic amines, stimulates adenylate cyclase and increases the intracellular concentration of cyclic AMP. Increases in cyclic AMP in turn may elevate phosphorylase a values and produce the inotropic effect (Robison et al. 1965).

It has also previously been suggested that the cardiac biochemical and mechanical effects of histamine are mediated through the elevation of cyclic AMP (Poch and Kukovetz 1967; Klein and Levey 1971; McNeill and Muschek 1972). Histamine, TD and betazole were found to produce a positive

inotropic effect and to increase cardiac phosphorylase in a dose-dependent manner. In the present study all of these compounds were found to elevate cardiac cyclic AMP. The order of potency and the order of effectiveness of the compounds in producing these effects was histamine>TD>betazole. The order obtained is the same as that previously found when the compounds were examined for their ability to activate cardiac adenylate cyclase (McNeill and Muschek 1972). Time-response relationships revealed that all three agonists elevated cyclic AMP prior to increasing contractility or phosphorylase a. The relative effectiveness of these drugs in increasing cardiac cyclic AMP was the same as for other parameters measured. A logical sequence of events for histamine and its analogs would appear to be: 1.) activation of adenylate cyclase, 2.) formation of cyclic AMP and 3.) enhancement of contractility and activation of phosphorylase a. It is difficult to separate the onset of contractility and phosphorylase activation with histamine. However when either TD or betazole was used, the activation of phosphorylase appeared to lag behind the positive inotropic effect. The specificity of the cardiac histamine receptor has been the subject of controversy. Several lines of evidence indicate that effects of histamine are independent of the adrenergic nervous system. Reserpine pretreatment did not alter the responsiveness of the heart to histamine (Trendelenburg, 1960, Mannaioni 1960). In fact reserpine-induced supersensitivity to the cardiac effects of histamine has been demonstrated (McNeill and Schulze 1972). Dichloroisoproterenol and propranolol did not abolish the histamine-induced changes in cardiac contractility and heart rate (Trendelenburg 1960; Pösch and Kukovetz 1967; McNeill and Muschek 1972). Dean (1968), reported that beta blocking agents like propranolol and prenethanol produced a slight shift in the histamine dose-response curve. This could be a non-specific antagonism. Hearts

isolated from animals pretreated with reserpine or 6-hydroxydopamine still show typical responses to the application of exogenous histamine (Levi and Giotti 1967). Such experiments demonstrate that histamine has direct cardiac effects.

Classical antihistamines such as diphenhydramine or tripeleminamine do not antagonize the responses of isolated cat and guinea pig atria and isolated perfused guinea pig hearts. (Trendelenburg 1960; Bartlett 1963; McNeill and Muschek 1972). These antihistamines were also relatively non-specific antagonists of the histamine stimulation of adenylate cyclase (McNeill and Muschek 1972). The principal reports claiming the demonstration of specific effects of antihistamine drugs in blocking the cardiac actions of low concentrations of histamine are those of Mannaioni (1960) and Flacke et al. (1967), Klein and Levey (1971), Hughes and Coret (1972) have reported that promethazine can antagonize the chronotropic effects of histamine on rabbit atria. Since complete dose-response curves were not obtained in that study, the specificity of the blockade is questionable. Black et al. (1972) have recently classified histamine receptors into H_1 and H_2 receptors. H_1 receptors mediate most effects of histamine and are blocked by classical antihistaminic compounds. Histamine stimulated secretion of acid from gastric mucosa and the inotropic and chronotropic effects of histamine can not be antagonized by mepyramine or related drugs. These receptors are classified as H_2 type receptors. Burimamide, however does block the gastric secretory and positive chronotropic effects of histamine (Black et al. 1972; Wylie et al. 1972). In the present study the specificity of the cardiac histamine receptor has been demonstrated.

Burimamide proved to be a competitive antagonist of the positive inotropic, phosphorylase activating and cyclic AMP increasing effects of histamine, TD

and betazole. Burimamide did not antagonize the effects of norepinephrine on these parameters and propranolol did not antagonize histamine or histamine analog cardiac effects. Stimulation of the adenylate cyclase then results in an increase in cyclic AMP, and subsequently a positive inotropic and phosphorylase activating effect similar to that suggested for catecholamines (Robison et al. 1965). Our data support the recent findings of Pöch and Kukovetz (1973), indicating that the inotropic as well as the cyclic AMP increasing actions of histamine can be inhibited by the H_2 antagonist burimamide.

The findings of Hughes and Coret (1972) suggest that the cardiac effects of histamine are susceptible to blockade by promethazine a H_1 receptor antagonist. A careful and complete study of the interaction of promethazine with several doses of histamine on heart rate, force of contraction and cyclic AMP formation was carried out to determine if the proposal of Hughes and Coret was correct. The data presented reveal that promethazine is not a competitive equilibrium antagonist of the cardiac effects of histamine but does interact with the histamine receptor in either a non-competitive or competitive non-equilibrium manner. Promethazine ($4-16 \times 10^{-6} M$) lowered the histamine effects on cardiac contractility. An increase in the dose of histamine could not overcome the promethazine blockade and increasing the promethazine concentration resulted in a further depression of the maximum histamine response. Promethazine also partially blocked the histamine-induced increases in cardiac cyclic AMP. Increases in the dose of histamine did not overcome the promethazine blockade. The decrease in the histamine response produced by promethazine was approximately the same for both the mechanical and biochemical events. Promethazine ($4 \times 10^{-6} M$) did not antagonise the inotropic response to norepinephrine.

Higher doses of promethazine ($8-16 \times 10^{-6} \text{M}$) did depress the norepinephrine responses. The results with promethazine stand in contrast to those obtained with burimamide. Burimamide appears to be a competitive equilibrium antagonist of histamine whereas promethazine exhibited characteristics of a non-competitive antagonist of histamine. One concentration of promethazine ($4 \times 10^{-5} \text{M}$) produced an increase in both cardiac contractility and rate. The stimulatory effects were not noted by McNeill and Brody (1968); Hughes and Cor  t (1972) or Davis and McNeill (1973). The effect was blocked by propranolol and this probably involved an adrenergic mechanism. Promethazine is known to block the uptake of adrenergic amines (McNeill and Brody, 1968; Davis and McNeill 1973). Such an affect could account for the results obtained.

Hughes and Cor  t (1972) have used only one concentration of promethazine ($4.7 \times 10^{-6} \text{M}$). In our study a complete range of promethazine concentrations ($2-16 \times 10^{-6} \text{M}$) and their interaction with histamine were investigated. The present study again demonstrated the importance of doing complete dose-response curves when investigating drug interactions.

The effect of histamine on adenylate cyclase was not blocked by propranolol and was poorly blocked by classical antihistamines such as tripeleennamine and diphenhydramine (McNeill and Muschek 1972). Our data indicate that the stimulatory effect of histamine and histamine analogs on guinea pig cardiac adenylate cyclase is competitively antagonised by burimamide. The interaction between burimamide and agonists was similar to that noted when the effects of these agents on cardiac cyclic AMP, contractility and phosphorylase were studied. The data presented differ from those of Klein and Levey (1971). They were able to demonstrate a complete blockade of one concentration of histamine with diphenhydramine

(8×10^{-5} M). However (10^{-5} M) diphenhydramine appeared to lower the maximum response to histamine (McNeill and Muschek 1972) and thus may again be an example of a non-competitive antagonism.

It has also been proposed that histamine produces its secretory effect in the gastric mucosa by interacting with H_2 receptors (Black et al. 1972). Cyclic AMP appears to act as an intracellular mediator of histaminic action on gastric mucosa, (Levine and Wilson 1971; Mao and Jacobson 1973; Bieck, Oates and Robison 1973). The stimulation of gastric adenylate cyclase by histamine, 4-methylhistamine, TD and betazole and their specific, competitive blockade by burimamide provide the basis for suggesting that histamine and its analogs stimulate cyclic AMP formation via an action on receptors in the membranes of the gastric mucosal cells.

The relative order of potency of histamine and its analogs on rat gastric adenylate cyclase is similar to that found in investigations studying the actions of these drugs on cardiac adenylate cyclase, cyclic AMP formation, phosphorylase activation and cardiac contractility (McNeill and Muschek 1972; present study). The rank order for the compounds is also similar to that noted when gastric acid secretion was measured (Lin et al. 1963). Karppanen and Westermann (1973) suggested that histamine stimulated gastric secretion of acid is mediated by cyclic AMP which is formed in response to stimulation of H_2 receptors. Our data agree with the findings of Dosa and Code (1974) that burimamide is a competitive antagonist of the stimulatory effects of histamine on gastric adenylate cyclase. The results are further supported by the findings of Narumi and Maki 1973; Bersimbaeve et al. 1971; Mao et al. 1973; Perrier and Laster 1970; who have provided evidence the H_2 receptor in gastric mucosa is associated with adenylate cyclase. Thus a logical sequence of events for histamine on gastric adenylate cyclase would

appear to be; 1.) activation of adenylate cyclase; 2.) formation of cyclic AMP; 3.) gastric acid secretion. Histamine, 4-methylhistamine, TD and betazole, all relaxed the rat uterus in a dose dependent manner. The order of potency of the compounds for relaxing the rat uterus was histamine > 4-methylhistamine > TD and betazole (Fig. 31). The present findings were in agreement with those of Black et al. (1972) who reported that histamine and 4-methylhistamine relaxed the rat uterus. Rat uterus possesses H_2 receptors as reported by Black et al. (1972). Stimulation of adenylate cyclase, prepared from guinea pig heart or rat stomach by histamine resulted in an increase in its activity and subsequently formed cyclic AMP. This data suggests that H_2 receptors in both the tissues were associated with adenylate cyclase.

However, we could not detect any changes in the adenylate cyclase activity by histamine. There could be two possibilities: 1.) H_2 receptors in the rat uterus are not associated with adenylate cyclase or 2.) our cyclic AMP method was not sensitive enough to pick up changes, if any, in cyclic AMP.

Recently, Tozzi (1973), reported that histamine effects on the rat uterus were through the release of catecholamines. If histamine effects on the rat uterus are not direct, then it would not stimulate adenylate cyclase prepared from rat uterus.

At least in two tissues, the heart and the rat stomach, H_2 receptors are associated with adenylate cyclase, stimulation of which results in an increase in cyclic AMP.

Histamine stimulates the guinea ileum by acting on H_1 receptors (Ash and Schild 1966). Burimamide did not block the histamine response on the guinea pig ileum. In the time-response study histamine (10^{-5} M) did not

increase cyclic AMP levels at any time tested. It thus appears that the effects of histamine on H_1 receptors are not mediated through stimulation of adenylate cyclase with a subsequent increase in cyclic AMP. These findings support the data of Black et al. (1972) that burimamide does not block the H_1 -receptor effects of histamine. Cyclic AMP levels are dependent on the activity of the enzyme phosphodiesterase. Theophylline inhibits phosphodiesterase while imidazole activates the enzyme in broken cell preparations (Butcher and Sutherland 1962). Both drugs have been used to provide indirect evidence for the involvement of the nucleotide in the positive inotropic response to catecholamines (Rall and West 1963; Kukovetz and Pösch 1967; McNeill 1970; McNeill and Muschek 1972).

The results presented here indicate that theophylline can produce an increase in cardiac contractility and phosphorylase a activation without elevating the levels of cyclic AMP. Theophylline also enhanced the cardiac effects of both histamine and norepinephrine without any apparent effect on the levels of cyclic AMP already elevated by norepinephrine and histamine. It has been assumed that because theophylline is a phosphodiesterase inhibitor it will elevate cyclic AMP levels in the intact tissue and thus produce its effects. The results of the present investigation indicate that this is not true for the heart. Earlier work with theophylline (Levey and Wilkenfeld (1968) using the rat uterus showed that theophylline potentiated the inhibitory response to nitroglycerin. Polacek et al. (1971) were able to dissociate the relaxant effect of theophylline on the rat uterus from any effect on cyclic AMP. Allen et al. (1973) have shown that there was no correlation within a group of phosphodiesterases inhibitors, including theophylline, between the phosphodiesterase inhibition and lipolysis in an isolated fat cell preparation. Some correlations have been found between various xanthine

derivatives with regard to their ability to inhibit phosphodiesterase and their interaction with norepinephrine on the heart (McNeill et al. 1973). The positive inotropic response to theophylline was not blocked by propranolol and it was therefore not mediated by catecholamine release (Massingham and Nasmyth 1972). Skelton et al. (1971) showed that theophylline could enhance the inotropic effect of norepinephrine and db. cyclic AMP but not of calcium on cat papillary muscle. They concluded that their data supported a role for cyclic AMP in the theophylline interaction. But Massingham and Nasmyth (1972) demonstrated a positive interaction between the electrical stimulation and theophylline in the frog ventricle. Such an effect would not be mediated through cyclic AMP and is more likely explained by an increase in the intracellular calcium. Methylxanthines have been found to release ionized calcium from the intracellular storage sites (Nayler 1963; deGubareff and Sleator 1965; Bianchi 1968; Shine and Langer 1971). This may be the process whereby xanthines increase myoplasmic calcium ions to cause a positive inotropic effect. (Nayler 1967). Caffeine, prolonged the active state of cardiac muscle (deGubareff and Sleator 1965; Gibbs 1967). Caffeine has been shown to markedly increase the duration of the action potential and to increase contractility about 45% in isolated atria. Epinephrine, on the other hand, increased contractility more than 100% and had little effect on action potential duration (deGubareff and Sleator 1965). The work of Blinks et al. (1972) has pointed out that the catecholamines decrease the time to peak tension and accelerate relaxation in cardiac muscle while the xanthines prolong time to peak tension and increase the total duration of contraction. Blinks et al. (1972) have reviewed the evidence suggesting that xanthines affect the heart by affecting calcium metabolism. Xanthines are known to increase the influx of calcium and to decrease the rate of calcium

sequestration in mammalian atria (Scholtz 1971; Shine and Langer 1971). Efflux of calcium is also reduced by caffeine (Shine and Langer 1971). More calcium is thus available for excitation contraction coupling and hence an increase in positive inotropic effect. The effects of xanthines on calcium may also explain the positive inotropic interaction of these drugs with biogenic amines on both contractility and phosphorylase activation, Namm et al. (1968), Stull and Mayer (1971) reported that calcium is essential for cardiac contractility and phosphorylase a activation.

Catecholamines and xanthines could produce their synergistic effect by elevating intracellular calcium by different mechanisms thus leading to an enhanced effect. The positive inotropic responses to theophylline can be interpreted in terms of the increased calcium influx which it produces. The interpretation of the effects of theophylline in terms of its action on phosphodiesterase should be treated with reservation. Methylxanthines thus appear to have direct effects on the heart, which are independent of their phosphodiesterase inhibiting properties.

Imidazole, a phosphodiesterase stimulator, appeared to produce positive inotropic effects on the heart which were not mediated through cyclic AMP. Poltetaeu (1970), reported similar effects of imidazole on skeletal muscle. Positive inotropic effects were seen when guinea pig hearts were perfused with imidazole (Knope et al. 1973). In their study they clearly showed that the imidazole-induced increases in myocardial contractility were not blocked by propranolol or antihistamines. Imidazole effects were not potentiated by aminophylline. Imidazole did not stimulate cardiac adenylate cyclase (McNeill and Muschek 1972). It thus seems unlikely that imidazole produces its inotropic effect by elevating cyclic AMP. This is further supported in the present investigations in that perfusion or injection of

imidazole did not produce any changes in cyclic AMP in the guinea pig heart.

Knope et al. (1973) suggested that the effect of imidazole was on tissue calcium or calcium turnover. DeMello et al. (1973) suggested that imidazole acted by increasing permeability to extracellular calcium in frog heart since responses to imidazole were totally suppressed in calcium-free media while those of caffeine were not.

Imidazole reduced the amine-induced increases in the levels of cyclic AMP as shown in Table 9 presumably by stimulating phosphodiesterase and thus increasing the metabolism of cyclic AMP. Imidazole stimulates the enzyme phosphodiesterase in vitro (Butcher and Sutherland 1962; McNeill et al. 1973). The decrease in cardiac cyclic AMP was paralleled by a corresponding decline in contractility. Pösch and Kukovetz (1967), reported that imidazole, in the Langendorff guinea pig heart preparation, caused a depression of cardiac contractility and an inhibition of the positive inotropic action of catecholamines. They attributed this effect to the stimulation of the phosphodiesterase-catalyzed breakdown of cyclic AMP. The lack of increase of cyclic AMP did not however, affect the ability of either amine to elevate cardiac phosphorylase a. Our data suggest the presence of a factor or factors other than cyclic AMP which are as important or more important, in activating cardiac phosphorylase. Friesen et al. (1967), reported an increase in cardiac phosphorylase by increasing external calcium in the perfusate. In hearts perfused with no calcium, norepinephrine injections did increase cardiac cyclic AMP, without any detectable changes in phosphorylase a (Namm et al. 1968). Their study suggested that calcium was still required for phosphorylase activation even when cyclic AMP was elevated. In smooth muscle calcium is of primary importance and cyclic AMP may not be required at all for phosphorylase activation (Diamond and Brody 1966; Namm 1971; Rasmussen et al. 1972). In a recent study, Diamond (1973) has shown increases

in phosphorylase activation during spontaneous uterine contractions at various times after increasing calcium concentration from 1.8 to 7.2mM. This further suggests the importance of calcium in phosphorylase activation without elevation of cyclic AMP. Similar conclusions can be drawn from experiments in electrically stimulated skeletal muscles in which phosphorylase activity but not cyclic AMP was found to be increased (Harwood and Drummond 1969; Posner et al. 1965). The recent demonstration by Stull and Mayer (1971) of an isoproterenol-induced increase in skeletal muscle phosphorylase a without an effect on cyclic nucleotide levels is a further illustration of this phenomenon. In all the references cited above, calcium has been invoked as the probable factor in activating the enzyme. A similar explanation would fit the data of the present study. In Table 11 a single injection of 1.6 mg imidazole alone was able to increase phosphorylase a. A recent study of McNeill and Young (1973) and Young and McNeill (1974), supports the concept that drugs can activate cardiac phosphorylase through mechanisms other than cyclic AMP. Hearts from hyperthyroid rats responded to norepinephrine to the same extent as controls when contractility and cyclic AMP were measured. However, phosphorylase activation by norepinephrine was enhanced in the hyperthyroid hearts. Hyperthyroidism is known to increase the accumulation and release of calcium in the heart (Suko 1971; Nayler et al. 1971). Again the involvement of calcium could explain the data.

Rasmussen and Tenenhouse (1970) have made several suggestions for the interaction of hormone, cyclic AMP and calcium. One of the suggestions is that hormone increases cyclic AMP, which in turn alters the permeability of the membrane to calcium. Our data support this suggestion. Both histamine and norepinephrine did not produce an inotropic effect in the imidazole

perfused hearts unless cyclic AMP was elevated. The phosphorylase activation, without any measurable changes in cyclic AMP, could be accounted for an increase in intracellular calcium. It is naive to think that a pharmacological agent has only one action. Theophylline or imidazole undoubtedly have many actions in addition to inhibition or stimulation of phosphodiesterase.

SUMMARY AND CONCLUSIONS

1. Phenylephrine increased cardiac cyclic AMP, contractility and phosphorylase a values in that order in the isolated perfused guinea pig heart. The effects of phenylephrine on the above biochemical and mechanical events were dose-dependent. Phenylephrine (in the doses used) is a weak beta-adrenergic agonist, being less potent and less effective than norepinephrine. The data are consistent with the hypothesis that adrenergic drugs produce their cardiac effects by stimulating adenylate cyclase and producing an increase in cyclic AMP.
2. (a) Histamine and the histamine analogs, TD and betazole, stimulated cardiac adenylate cyclase and increased cardiac cyclic AMP levels. The drugs also increased contractility and phosphorylase a.
 (b) Histamine was most potent as compared to its analogs, TD and betazole in increasing cardiac contractility, phosphorylase a and cyclic AMP. The order of potency observed was histamine > TD > betazole.
 (c) Burimamide competitively antagonized the cardiac effects of histamine and its analogs. Burimamide showed its specificity for histamine by not affecting any of the norepinephrine-induced cardiac effects, namely contractility, phosphorylase and cyclic AMP elevation.
3. Promethazine, a H_1 receptor antagonist also blocked the cardiac effects of histamine, including the histamine-induced increases in cyclic AMP. The blockade of cardiac histamine effects caused by promethazine was of a noncompetitive or noncompetitive nonequilibrium type. Promethazine ($4 \times 10^{-6} M$) produced inotropic effects but at a higher concentration ($8-16 \times 10^{-6} M$) was cardiodepressant. Promethazine ($4 \times 10^{-6} M$) did not

affect the positive inotropic response to norepinephrine. At $8-16 \times 10^{-6} \text{ M}$ promethazine lowered the maximum response of norepinephrine.

4. Histamine and its analogs, 4-methylhistamine, TD and betazole stimulated cardiac adenylate cyclase. The order of potency of the compounds for stimulating cardiac adenylate cyclase was histamine > 4-methylhistamine > TD > betazole. Stimulation by the agonists was blocked, in an apparently competitive manner, by burimamide.
5. Histamine, 4-methylhistamine, TD and betazole stimulated the gastric adenylate cyclase, over a dose-range of $10^{-6} - 10^{-2} \text{ M}$. The rank order of stimulation was histamine > 4-methylhistamine > TD and betazole. Burimamide ($1-10^{-6} \text{ M}$) antagonized the effect of the drugs in an apparently competitive manner.
6. Histamine, TD and betazole relaxed the estrogen primed rat uterus. The order of relaxation was histamine > 4-methylhistamine > TD and betazole. However, we failed to detect any changes in the adenylate cyclase activity due to histamine interaction.
7. Histamine in a dose of 10^{-5} M produced a maximum effect on the isotonically contracting guinea pig ileum. Burimamide did not block the histamine effect on the guinea pig ileum. Histamine at no time increased the levels of cyclic AMP over control, when measured in the frozen tissues.

The present data are consistent with the hypothesis that histamine and its analogs produce their action by stimulating adenylate cyclase and elevating cyclic AMP. H_2 receptors appear to be associated with adenylate cyclase, at least in two tissues, the heart and the stomach.

Histamine receptors in guinea pig ileum are of H_1 type. Stimulation of H_1 receptors does not increase cyclic AMP and hence H_1 receptors are not associated with adenylate cyclase.

8. The methylxanthine derivative, theophylline potentiated the cardiac effects of norepinephrine or histamine. Theophylline enhanced the inotropic and phosphorylase activating effects of both amines. Theophylline, when injected into the heart, produced an inotropic effect and phosphorylase a activation but did not elevate cyclic AMP. The data presented suggest that cardiac effects of methylxanthines are not mediated through cyclic AMP, but are more readily explained by the effects of these agents in calcium metabolism.
9. Imidazole, a phosphodiesterase stimulator, lowered the levels of cyclic AMP in the heart following the injection of histamine or norepinephrine and also decreased the inotropic effect of these agents. Imidazole however did not affect the phosphorylase activating effect of other amines, suggesting that mechanisms other than cyclic AMP, presumably calcium, are as important for activating the enzyme in cardiac muscle as they are in smooth and skeletal muscle. However data obtained with imidazole suggest that cyclic AMP is required for amine-induced increases in cardiac contractility.

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APPENDIXREAGENTS USED FOR PHOSPHORYLASE ASSAY:

I 10% TCA

II Fiske and Subbarow reagent, It contains:

1-amino-2-naphthol-4-sulfonic Acid	500 mg
Na_2SO_3	1.0 gm
NaHSO_3	30.0 gm
Dist. Water	to make 200.0 ml

Na_2SO_3 and NaHSO_3 were mixed in 120 ml of distilled water, and to this added, with constant stirring, sulfonic acid. The solution was stirred for several hours. It was filtered and stored protected from light.

III TRIS. buffer (Sigma 7-9 standard and Biochem buffer)

pH, adjusted to 6.9, using concentrated HCl.

IV Shelf Molybdate Stock Solution.

Working solution of shelf molybdate was made fresh from stock solution by appropriate dilution with distilled water.

V Glycogen 4.0%

The glycogen solution was made by passing through the Dowex-column which was previously treated with hydrochloric acid and washed free of chloride.

REAGENTS USED FOR CYCLIC AMP BINDING ASSAY:

I Sodium acetate 100mM.

II Potassium phosphate buffer 20mM.

III 5% Trichloroacetic Acid

IV 1N-HCl

V Cyclic AMP dependent protein kinase 3.2 $\mu\text{g}/30\lambda$

VI Protein kinase inhibitor (from beef heart) 1.1 mg/ml

VII Unlabelled cyclic AMP 200 p moles (10^{-12} moles)/ml

VIII $3[\text{H}]$ cyclic AMP 200 p moles/ml

REAGENTS USED FOR ADENYLATE CYCLASE ASSAY:

I Tris buffer 0.3M

II Sodium fluoride 0.06M

III Theophylline 0.06M

IV KCl 0.166M

V $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.45M

VI Salt mix: KCl, MgSO_4 , in 1:1 ratio

VII Phosphoenol pyruvate 0.3M

VIII ATP 5mM

IX Pyruvate kinase, 1 to 5 dilution (freshly prepared).

LIQUID SCINTILLATION COCKTAIL

PPO	4.0 gm
POPOP	50.0 mg
Toluene to make	1000 ml

COMPOSITION OF THE HEART PERFUSION FLUID: (Stock Solution)

(Chenoweth and Koelle, 1946)

Sodium chloride	140.0 gm
Dextrose	36.0 gm
Pot. chloride	8.4 gm
Ca.Cl ₂ .2H ₂ O	6.4 gm
MgCl ₂ .6H ₂ O	8.6 gm
Dist. water to make	2000 ml

The working Chenoweth and Koelle buffer was made by dilution 200 ml. of the stock solution to 1000 ml with distilled water. The pH was adjusted with sodium bicarbonate. The perfusion fluid was aerated with mixer of gases, 95% O₂ + 5% CO₂.

COMPOSITION OF THE BATHING SOLUTION FOR THE RAT UTERUS: (Diamond, 1973)

1. Sodium Chloride Tris buffer:

Chemical	m mol./litre
Sodium chloride	125.0
Potassium chloride	2.4
Magnesium chloride	0.5
Glucose	11.0
Tris*	23.8
Calcium chloride	1.8

2. Potassium Chloride Tris buffer:

<u>Chemical</u>	<u>m mol./litre</u>
Potassium chloride	127.4
Magnesium chloride	0.5
Glucose	11.0
Tris*	23.8
Calcium chloride	1.8

*Tris Tris(hydroxymethyl)-aminomethane

All buffers were adjusted to pH 7.4 with concentrated HCl, and aerated with 100% oxygen.

COMPOSITION OF THE TYRODE SOLUTION FOR THE GUINEA PIG ILEUM: (All solutions are expressed in mM).

NaCl, 136.89; KCl, 2.68; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 1.76; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.98; $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$, 0.36; NaHCO_3 , 11.90; Glucose, 5.55; distilled water to make 1000 ml of the solution.

The tyrode solution was adjusted to pH 7.4 and aerated with 95% O_2 and 5% CO_2 .

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