EFFECTS OF ETHANOL ON GROWTH AND CYCLIC AMP IN CULTURED CELLS

by

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SUMMARY

EFFECTS OF ETHANOL ON GROWTH AND CYCLIC AMP IN CULTURED CELLS

Studies on the effects of ethanol on the body have so far failed to identify any direct mechanism of action that may account for the typical pattern of alcohol intoxication *in vivo*. The ethanol molecule is capable of reaching the cell membrane of any cell in the body and is known to cause ionic and physical alterations in membrane structure *in vivo*. The intracellular molecule adenosine 3',5' - cyclic monophosphate (cyclic AMP) has a postulated role as a mediator of hormone action. The formation of cyclic AMP inside the cell is catalysed by the enzyme adenyl cyclase, which is positioned on the cell membrane. It has been suggested that some of the *in vivo* effects of ethanol may be related to cyclic AMP metabolism.

To investigate the action of ethanol at the cellular level, the growth pattern of cultured cells was examined in the presence of ethanol. Concentrations of ethanol were similar to those found in the blood stream of patients admitted intoxicated to the Western Infirmary. L929 cells, a cultured cell line originally derived from mouse fibroblasts, were used. They were regarded as a feasible model for study of intracellular cyclic AMP changes since any such changes are likely to be reflected by alterations in the growth rate of the cells. Growth parameters including cell population density, DNA, RNA and protein were monitored also.

Measurement of intracellular cyclic AMP involved establishing an adequate protein binding assay. The validity of the assay was confirmed in terms of quantitative values for assay specificity, accuracy, precision and sensitivity.

Ethanol treatment was found to delay cell division probably by temporarily preventing entry into the S phase of the cell cycle. This event was parallelled by higher cell cyclic AMP in ethanol treated cells. Experimental cells tended to be poor in content of DNA and RNA; protein results implied a relationship between cell protein, cell cyclic AMP and growth rate. The evidence suggested that elevated cell cyclic AMP was a critical, though not necessarily primary event related to decreased growth, and could have been a response to decreased availability to the cell of crucial nutrients.

After approximately 24 hours delay, ethanol treated cells grew normally in a logarithmic fashion. That the cyclic AMP molecule may have played a role in this compensatory mechanism, is discussed. It is suggested that the same molecular mechanisms may be involved <u>in vivo</u>.

Further experiments are suggested to examine the specificity of the alcohol interference and to verify the presence of a compensatory mechanism involving enzyme induction.

INTRODUCTION

EFFECTS OF ALCOHOL ON THE BODY

Among the drugs of abuse, none has achieved such wide popularity as ethanol. Compared with other psychoactive drugs, the molecular size of ethanol is small. It is, under usual conditions, rapidly absorbed through the mucosal membranes of the gastrointestinal tract (especially the duodenum and jejunum) and enters the portal circulation. Distributed through the body in the arterial blood, the molecule diffuses rapidly across capillary membranes. It moves across tissue membranes by a process of simple diffusion (Kalant 1971). Within a few minutes after oral ingestion, it has been circulated to every tissue of the body. Its movement is not blocked by the "blood-brain barrier" or the "placental barrier". In view of this complete distribution throughout the body and into individual cells, one can readily appreciate the fact that a wide range of cellular functions such as membrane permeability, transport mechanisms and intracellular enzyme systems may be affected by ethanol.

Scientific knowledge of the acute and chronic effects of ethanol is far from complete. The problem is one of considerable complexity due to the unique dual nature of the action of this compound in the body. It is, on the one hand, a central nervous system (CNS) depressant of the general anaesthetic type and on the other, a nutrient whose rapid oxidation in the cell can cause marked disruption of normal metabolic processes in peripheral metabolism (Seixas 1975).

Acute Effects

With reference to immediate (acute) effects of alcohol, most users experience a fairly reproducible sequence of effects (Kalant 1970) which, depending on dosage and past experience, includes the following: reductions in sensory acuity; decreased span of attention to environmental stimuli; loss of inhibition of emotional expression (eg talkativeness in social settings); progression of drowsiness, sleep and coma at higher doses; a series of autonomic changes mediated through the hypothalamus resembling those that occur during sleep, such as increased peristalsis, increased gastric secretion; depressed secretion of vasopressin, oxytocin and gonadotrophic hormones, and disturbances of modulatory control of proprioceptor and motor pathways.

Ethanol and the CNS

In the experimental and clinical study of alcoholism, it is generally assumed that those effects that are produced by a direct action of ethanol on neurons of the CNS are most likely to be the initiators of tolerance and dependence. The inhibitory effect of ethanol on $(Na^+ + K^+)$-stimulated adenosine triphosphatase activity and on active transport of cations across neuronal membranes (Kalant and Israel 1967; Israel 1970) has been verified by several groups of investigators (Sun and Samorajski 1970; Nikander et al 1971; Goldstein and Israel 1972). It is not surprising that active transport mechanisms for other substances which are linked

to simultaneous transport of Na^+ and K^+ are also inhibited by ethanol. These include uptake of various putative neurotransmitters, especially glutamine, into synaptosomes (Roach et al 1973) and uptake of ^{3}H-lysine (Choy et al 1972) and α-aminoisobutyrate (Freud 1972) into brain in vivo.

Since passive ion fluxes during action potentials and active fluxes during recuperation between impulses are basic to all neuronal activity, one might reasonably expect all other metabolic indices of cell activity to be affected by ethanol inhibition of these two processes. Decreased brain protein turnover (reviewed by Noble and Tewari 1975), reduced intermediary metabolism and high energy phosphate turnover (Veloso et al 1972), decreased incorporation of ^{32}P into phospholipid (Brossard and Quastel 1963) and decreased cyclic AMP levels only after massive acute doses of ethanol (Kuriyama and Israel 1973; Volicier and Gold 1973) may all be simply reflections of decreased neuronal activity.

Paradoxically, chronic ethanol ingestion leads to a differential effect on brain proteins, including an increase in the synthesis of some proteins (Noble and Tewari 1975) and as such could influence proteins that may be critical for brain function. Furthermore chronic ethanol treatment produces adrenergic sensitivity, in particular with relation to the beta-receptor effects (cyclic AMP formation) of noradrenalin in brains of experimental animals. (French and Palmer 1973). Withdrawal reactions from alcohol (Victor 1970) include catecholamines (Pohorecky et al 1974). Genetic factors

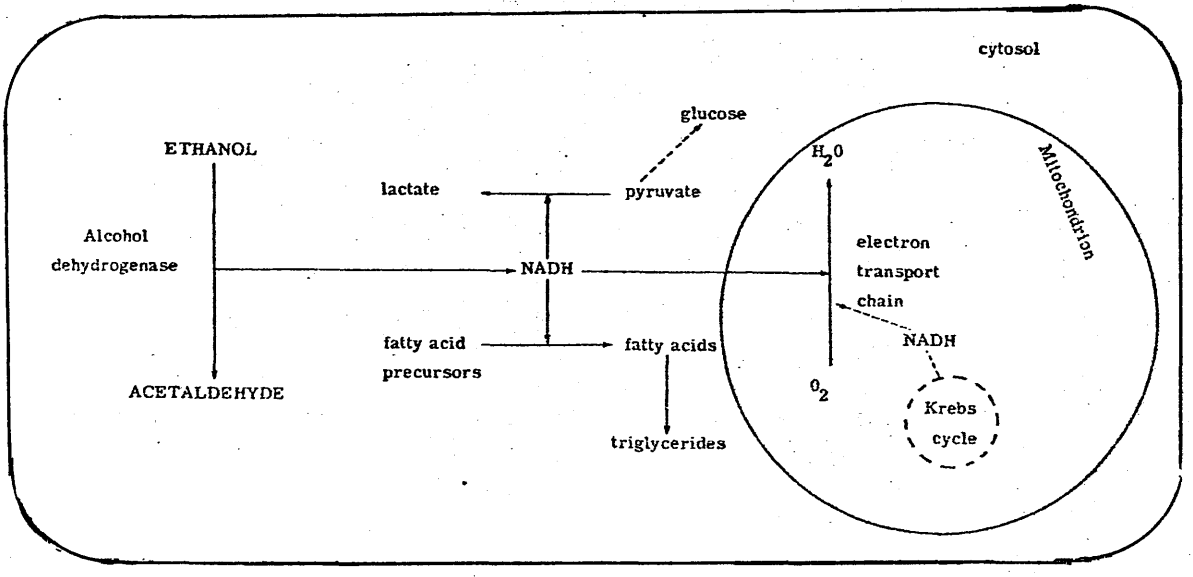
appear to be involved (Partanen et al 1966), although it is not known whether the inherited factors affect the tendency to drink or the susceptibility to addiction with a given alcohol intake.

Ethanol and Peripheral Metabolism

Of the many visceral changes to which alcohol is related, liver injury as an acute or chronic process is predominant. It is well established that about 90% of ingested ethanol is metabolised in the liver (Jacobsen 1952). Hepatic metabolism of ethanol to acetaldehyde by the alcohol dehydrogenase pathway (Fig 1) is associated with the generation of reducing equivalents as NADH. Several cytoplasmic and mitochondrial functions are then altered due to the lowered redox state produced by the oxidation of ethanol (Williamson et al 1969; Hawkins and Kalant 1972), eg oxidation of fatty acids (Lieber et al 1967), the citric acid cycle (Lieber 1968; Williamson et al 1969). This explains a variety of effects following acute ethanol administration, including enhanced lipogenesis and depressed lipid oxidation.

Following chronic ethanol consumption, adaptive microsomal changes prevail, which include enhanced ethanol and drug metabolism and increased lipoprotein production. Several hepatic lessions (alcoholic hepatitis and cirrhosis) develop. Chronic ethanol consumption is associated with ultrastructural changes in the mitochondria (Iseri et al 1966; Rubin et al 1970), even in organs in which ethanol is not oxidised, eg the pancreas (Darle et al 1970), the small intenstine (Rubin et al 1972) and skeletal muscle (Song and Rubin 1972).

FIGURE 1



Metabolism of ethanol in the liver cell by alcohol dehydrogenase and some biochemical pathways which are enhanced. Pathways which are decreased by ethanol are represented by dashed lines.

Current knowledge of acute, chronic, tolerance and withdrawal effects of alcohol has been reviewed by Dietrich (1975) and Smuckler (1975). The former review is directed towards effects of ethanol on the CNS. The latter is concerned with more peripheral responses, including molecular and hormonal changes.

CYCLIC AMP

Adenosine 3',5' - monophosphate or "cyclic AMP" (c AMP) is present in all types of mammalian cells under physiological conditions, constituting less than 0.1% of the adenine nucleotide pool. The cyclic nucleotide does not participate as an intermediate or coenzyme in metabolic pathways, but plays a role in the regulation rather than maintenance of cellular activities (Robison et al 1971).

The production of cyclic AMP in the cell is controlled by the enzyme adenyl cyclase (AC) (Sutherland et al 1962) which is a component of the cell membrane, or of membranous structures within the cell (Sutherland et al 1962; de Robertis et al 1967, Rabinowitz et al 1965). Another enzyme, found principally in the soluble fraction of the cell, cyclic nucleotide phosphodiesterase (PDE), hydrolyses cyclic AMP to 5' - adenosine monophosphate (Butcher and Sutherland 1962).

The Second Messenger Hypothesis of Hormone Action

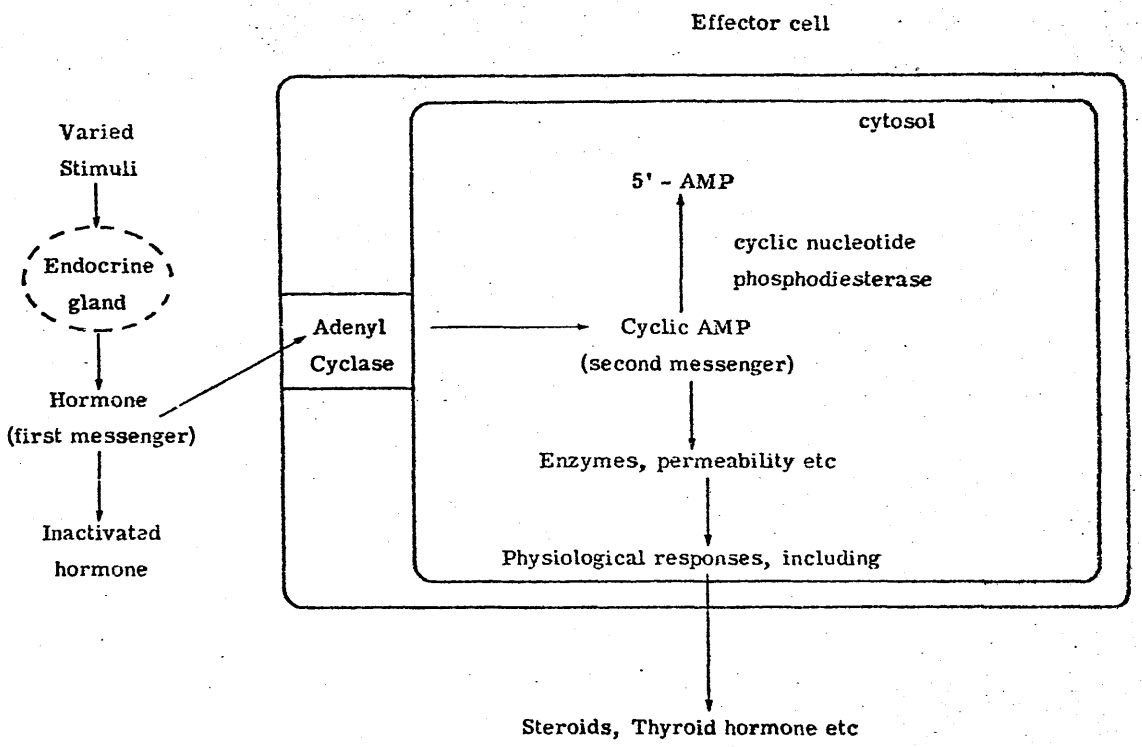
Sutherland's group and other investigators have demonstrated that many polypeptide hormones increase the concentrations of cyclic AMP, or adenyl cyclase activity in target tissues (Table I) These findings led to the "two messenger hypothesis" of hormone action (Fig 2 ; Sutherland et al 1965). The hormone is the first messenger; it circulates in the blood, binds to the plasma membrane of the target cell and activates adenyl cyclase. Cyclic AMP, the second messenger, is generated on the inner surface of the cell membrane and brings about the appropriate physiological response within the cell. While this model may or may not be correct, it adequately describes the functional state of adenyl

TABLE I

Hormones which affect Cyclic AMP

Hormone	Tissue	Principal Responses	Reference
Adrenalin	Liver	Glycogenolysis	Sutherland and Rall (1960)
	Muscle	Glycogenolysis	Sutherland and Rall (1960)
	Heart	Inotropic Effect	Skelton et al (1970)
	Salivary Gland	Amylase Secretion	Bdolah and Schramm (1965)
	Fat	Lipolysis	Butcher et al (1965)
Noradrenalin	Brain	Discharge frequency of Purkinje cells	Siggins et al (1969)
	Nerve	Acetylcholine release	Goldberg and Singer (1969)
	Pineal	Melatonin synthesis	Shein and Wurtman (1969)
Glucagon	Liver	Glycogenolysis	Sutherland and Rall (1960)
	Fat	Lipolysis	Butcher et al (1968)
Thyroid Stimulating Hormone	Thyroid	Thyroglobin hydrolysis	Pastan and Wollman (1967)
		Iodination	Ahn and Rosenberg (1968)
		Glucose oxidation	Pastan and Macchia (1967)
Parathyroid Hormone	Renal Cortex	Phosphaturia	Chase and Aurbach (1967)
	Bone	Calcium resorption	Chase and Aurbach (1967)
Adrenocorticotrophic Hormone	Adrenal	Steroid production	Haynes et al (1959)
	Fat	Lipolysis	Butcher et al (1968)
Luteinising Hormone	Corpus Luteum	Steroid synthesis	Marsh et al (1966)
Vasopressin	Renal Medulla	Water resorption	Orloff and Handler (1967)
Thyroxine	Heart	Tachycardia	Levey and Epstein (1969)
Angiotensin	Adrenal Cortex	Aldosterone production	Kaplan (1965)

FIGURE 2



The second messenger hypothesis of hormone action.

cyclase. Fig 3 depicts the hypothetical representation of the adenyl cyclase molecule as proposed by the second messenger hypothesis.

Breakdown of Glycogen

In only once case - the control of glycogen breakdown - is there sufficient information to explain at a chemical level how cyclic AMP acts. The regulation of glycolysis has been postulated to serve as a model for the mechanism of action of cyclic AMP (Krebs et al 1966; Walsh et al 1968). In this model (Fig 4) cyclic AMP stimulates a protein kinase which catalyses phosphorylation of another functional protein. Phosphorylation reversibly alters the conformation and subsequently the function of intracellular proteins.

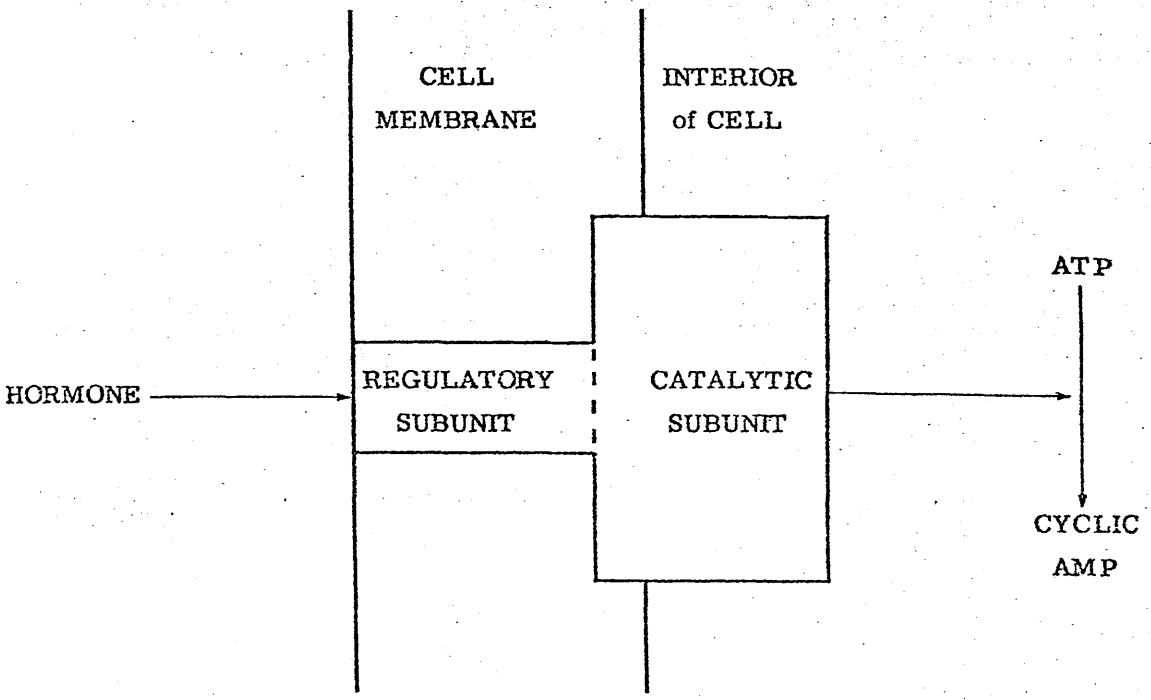
Cyclic AMP and Histones

The ability of cyclic AMP dependent protein kinases to phosphorylatehistone, the basic protein associated with DNA (Langan 1969) confers a role to cyclic AMP in regulation of transcription, as found in bacteria (Varmus et al 1970). It has been proposed (see Langan 1969) that the increased histone phosphorylation brought about by hormone administration might provide a mechanism for induction of RNA and protein in target tissues. However the mechanisms controlling gene expression in higher animals are as yet obscure (see Rasmussen and Bordier 1974).

Effects of Ions

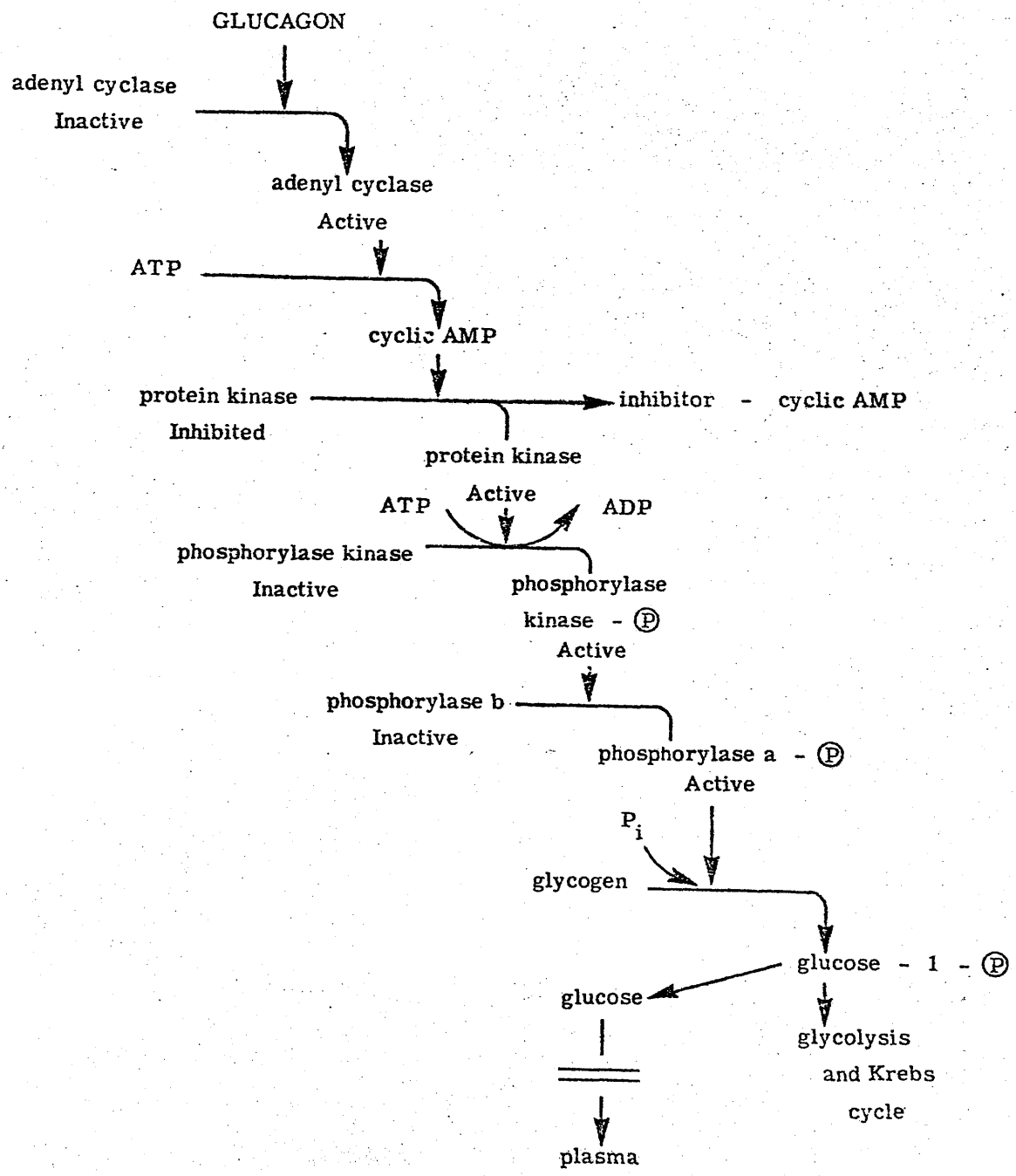
Adenyl cyclase has been reported to be influenced by local ionic changes, eg K^+ has been shown to increase cyclic AMP levels in brain slices (Sattin and Rall 1967) and Ca^{2+} is known to be a requirement of ACTH (Adrenocorticotrophic hormone) stimulation of adenyl cyclase activity in the adrenal cortex (Bar and Hechter 1969).

FIGURE 3



Hypothetical representation of the adenyl cyclase molecule as proposed by Robison et al (1967). According to this model, the hormone would interact with the regulatory subunit, which in turn would influence the configuration of the catalytic subunit.

FIGURE 4



Regulation of degradation of glycogen in response to Glucagon.

The particulate nature of adenyl cyclase, together with its lability, especially as regards hormonal sensitivity, have combined to make the purification and study of this enzyme difficult. Although it has been extensively investigated since its discovery in 1960, the hypothetical representation of the adenyl cyclase molecule proposed by Robison et al (1967) has not yet become a reality.

The only evidence of cyclic nucleotide phosphodiesterase activity being altered, apart from inhibition by ATP (Cheung 1967), is by a variety of drugs <u>in vitro</u> (Hess et al 1975).

EFFECTS OF ETHANOL ON CYCLIC AMP

The physiological, biochemical and neurological processes affected by alcohol to which cyclic AMP is believed to be related are ennumerate. Outwith the cell, alcohol is known to immediately affect neuronal excitability, impulse induction, neurotransmitter and catecholamine release. At the cell membrane, it is known to affect Na^+ and K^+ transport. Within the liver cell, the metabolism of alcohol interferes with gluconeogenesis and lipid oxidation. Tolerance to (and physical dependence on) alcohol involve compensatory changes, resulting in "adrenergic supersensitivity" (French and Palmer 1973), increased $(Na^+ + K^+)$-ATPase, enhanced oxidative phosphorylation. When alcohol ingestion is abruptly discontinued, the compensatory changes are maladaptive and give rise to the basic symptoms of the withdrawal reaction, eg, hyperflexia, muscle tension and anxiety; the stress responses involving elevations in plasma and urinary catecholamines (see reviews, Dietrich 1975; Smuckler 1975).

The position of adenyl cyclase on the cell membrane and the role of cyclic AMP as a biochemical "trigger" through which a physiologic event - the release or action of a hormone, is translated to a biochemical event within the cell (eg glycogenolysis, lipolysis) suggests that some molecular action, involving the cell membrane and cyclic AMP, may be involved in at least some of the effects of ethanol on the body.

Cell Membrane Alterations by Ethanol

The strongest evidence to date regarding cellular mechanisms of action of ethanol concerns biophysical alterations in membrane properties. Ethanol in sub-lethal concentrations has been found to have an inhibitory effect on the active transport of cations in widely diverse tissues, eg the isolated frog skin (Israel and Kalant 1963), red blood cells *in vivo* (Lindsay 1974a) slices of guinea pig brain cortex and rat renal cortex (Israel-Jackard and Kalant 1965), canine gastric mucosa *in vivo* (Rehm and Hokin 1947). Thus the effect occurs even in tissues where no depolarisation is known to take place normally. Changes in the ionic environment surrounding intact cells are known to have a profound effect on the intracellular level of cyclic AMP (Robison et al. 1971) as well as adenyl cyclase (page 7).

Meier and Mendoza (1976) have reported a decreased cyclic AMP response to vasopressin in the toad bladder *in vitro* due to a decrease in the resting potential produced by a massive dose of ethanol. The degree to which muscle phosphorylase *in vitro* is activated by adrenalin has been shown to be dependent upon the ratio of extracellular Na^+/K^+. Gluco-corticoids, suggested to be a vital part of the mechanism which maintains intra- and extracellular electrolyte balance of vertebrate organisms (Swingle et al 1960), may support the actions of catecholamines *in vivo* by providing the correct ionic environment (see review, Brodie et al 1966).

The principal effect of cyclic AMP is to stimulate glycogenolysis, lipolysis and steroid production via the

actions of peptides and thyroid hormones and the adrenergic system (Table I). Acute administration of adrenalin to rats produces, after 1 - 2 hours, a calorigenic effect (increased rate of oxygen consumption) in the liver identical to that seen after thyroxine treatment or after chronic treatment with ethanol (Israel et al 1973).

This "liver hypermetabolic state" (Bernstein et al, 1974) when induced by ethanol, was blocked in adrenalectomised rats (Israel et al 1975), implying that an increase in adrenergic tone was involved. A number of similarities can be observed in the liver as a result of administration of thyroid hormones and chronic treatment with ethanol (Israel et al 1973). Among these is the fact that liver necrosis and cirrhosis had occurred in 50-60% of the patients who died of severe thyrotoxicosis before the advent of modern therapy (see Bernstein et al 1974).

In the liver, evidence has been given for the possibility that effects of ethanol, adrenergic agents and thyroid hormones are exerted at different levels of a common sequence of events leading to stimulation of the $(Na^+ + K^+)$-ATPase and consequently increasing oxygen consumption (Israel et al 1975).

Thyroid hormones have primary functions in regulating protein synthesis (Tata 1963; Weiss and Sokoloff 1963). Brodie et al (1966) reported that thyroid hormones caused an increase in synthesis of adenyl cyclase in adipose tissue, rendering it more susceptible to the effects of noradrenalin. The turnover rate of noradrenalin is similar in normal and hyperthyroid mice (Beaven et al 1963), implying that thyroid

hormones do not increase the rate of noradrenalin synthesis. It is reported that noradrenalin content in the brain does not change following chronic ethanol ingestion (Pscheidt et al 1961). In the brain, the site of "adrenergic supersensitivity" produced by ethanol is believed to be at the level of cortical receptors (French and Palmer, 1973; French et al 1975).

The mechanism of adrenergic supersensitivity is controversial: for example, Fleming et al (1973) related it to non-specific changes in the postjunctional membrane, whereas Deguchi and Axelrod (1973) reported a change in the response of the specific receptor for noradrenalin.

The evidence to date does not dispute the possibility that the same type of molecular action, involving the cell membrane and cyclic AMP may be involved in all of the effects of ethanol on the body.

Direct Effects of Ethanol on Cyclic AMP

Exploration of any direct effects of ethanol on mechanisms involving cyclic AMP have so far provided conflicting or unconclusive results. (Tables II and III). For example, Gorman and Bitensky (1970) who examined the in vitro effects of ethanol on adenyl cyclase in rat liver homogenates, reported activation of adenyl cyclase by ethanol which was further enhanced by glucagon yet unaffected by adrenalin. Their results suggested a reversible, conformational change in the adenyl cyclase molecule itself. However Mashiter et al (1974) found that ethanol had no effect on adenyl cyclase in homogenates, yet a stimulatory one in whole cell preparations and suggested that ethanol could activate adenyl cyclase as a result of a non-specific perturbation of membrane structure. Conflicting

TABLE II

Reported Effects of Ethanol on Cyclic AMP Metabolism in vitro

Tissue	Species	% Ethanol Concentration	Effect on AC Activity	Effect on PDE Activity	Comment	Reference
Liver homogenate	rat	5	stimulated	n.m.*	Potentiated by glucagon	Gorman and Bitensky (1970)
Liver homogenate	rat	11	stimulated	n.m.	Additive effect with fluoride – Stimulated AC at 11% ethanol	Greene et al (1971)
Liver homogenate	rat	0.25-5	no effect	n.m.	—	Mashiter et al (1974)
Liver whole cell	rat	0.25-5	stimulated	n.m.	—	Mashiter et al (1974)
Perfused liver	rat	1	no effect	n.m.	—	Jauhonen et al (1975)
Kidney homogenate	rat	0.2-33	stimulated	n.m.	Additive effect with fluoride – Stimulated AC at 11% ethanol	Greene et al (1971)
Kidney homogenate	rat	0.25-5	no effect	n.m.	—	Mashiter et al (1974)
Kidney whole cell	rat	0.25-5	stimulated	n.m.	—	Mashiter et al (1974)
Fat homogenate	rat	0.2-33	stimulated	n.m.	—	Greene et al (1971)
Thyroid homogenate	beef	0.25-5	no effect	n.m.	—	Mashiter et al (1974)
Thyroid whole cell	beef	0.25-5	stimulated	n.m.	—	Mashiter et al (1974)
Thyroid whole cell	dog	0.25-5	no effect	n.m.	—	Mashiter et al (1974)
Gastric mucosa homogenate	rat	20	inhibited	inhibited	Fluoride – stimulated AC	Tague and Shanbour (1974)
Intestine homogenate	rat	0.2-33	stimulated	n.m.	—	Greene et al (1971)
Brain homogenate	rat	0.2-33	stimulated	n.m.	Additive effect with fluoride – Stimulated AC at 11% ethanol	Greene et al (1971)
Brain homogenate	mouse	0-2.5	no effect	no effect	—	Kuriyama and Israel (1973)

* n.m. not measured

TABLE III

Reported Effects of Ethanol on Cyclic AMP Metabolism in vivo

Tissue Investigated	Species	Duration of Ethanol Subjection	Effect on AC Activity	Effect on PDE Activity	Effect on Cyclic AMP Levels	Comment	Reference
Liver	Rat	hours	n.m.*	n.m.*	increased	—	Jauhonen et al (1975)
Adipose tissue	Rat	hours	n.m.	n.m.	no effect	—	Jauhonen et al (1975)
Gastric mucosa	Rat	mins	decreased	n.m.	no effect	Fluoride stimulated AC. Ethanol administered via a stomach tube	Tague and Shanbour (1974)
Gastric mucosa	Rat	mins	decreased	decreased	decreased	Ethanol administered intravenously	Puurunen and Karppane (1975)
Brain	Rat	mins – hours	n.m.	n.m.	decreased	Ethanol administered via a stomach tube	Volicier and Gold (1973)
Brain	Mouse	mins – hours	no effect	no effect	no effect	Ethanol administered intravenously	Kuriyama and Israel (1973)
Brain	Mouse	1–3 weeks	increased	no effect	increased	No net increase over control when fluoride present	Kuriyama and Israel (1973)
Brain	Mouse	2 weeks	increased	no effect	increased	Loss of responsiveness to noradrenalin in ethanol treated mice	Israel et al (1972)

* n.m. not measured

results are not restricted to liver: ethanol stimulated adenyl cyclase in beef thyroid slices *in vitro* but not in dog thyroid slices (Table II); acute alcoholic intoxication of rats *in vivo* decreased brain cyclic AMP yet had no effect on cyclic AMP levels in brains of mice (Table III). These results indicate that species differences may lead to differing dose effects.

Differences in rate and route of administration of ethanol have complicated interpretation of results: whereas ethanol administered intravenously to rats decreased gastric mucosal cyclic AMP levels, it has been reported to have no effect on gastric mucosal levels of cyclic AMP in rats, when administered via a stomach tube (Table III).

Experimental Complications

Adenyl cyclase has proven to be a technically difficult model for investigations at the molecular level, its particulate nature making it susceptible to destabilisation. Methodology has been further complicated by the three hundred-fold greater activity of phosphodiesterase relative to adenyl cyclase (Tague and Shanbour 1974). Fluoride has been added to ethanol-treated systems to activate adenyl cyclase, an additive effect suggesting that ethanol and fluoride initiate the cyclic AMP response through specific "receptor" sites (Table II). However, fluoride has been shown to activate adenyl cyclase by release of an inhibitory subunit, and only in homogenates (Perkins and Moore 1971).

In all of the *in vitro* experiments to date, the effects of ethanol were maximal at concentrations well above those that are meaningful *in vivo*. The breathalyser limit is 80 mg ethanol per 100 ml blood (0.10%); concentrations above 0.5% are fatal (Smuckler 1975). Ethanol moves freely through the body by

simple diffusion - only in the alimentary tract of a fasting individual could its concentration be elevated much above 5%. The ability of ethanol to denature proteins at concentrations above 10% has been recognised for many years.

The interpretation of any in vivo findings to date are complicated by the fact that functional alteration of any in vivo system by ethanol may reflect: direct local actions of ethanol on the cells under study; a change in nervous or chemical input to these cells because of an action elsewhere in the body; effects of ethanol metabolites; disturbances in homeostasis in the whole body as a result of deep intoxication and/or experimental trauma.

GROWTH OF CELLS IN CULTURE

Animal cell culture is concerned with the study of cells explanted from animals and maintained *in vitro* for more than twenty-four hours. (Paul 1970). Many vertebrate tissues can be disrupted into individual cells, (for example by digestion with trypsin.), which will propagate at a temperature of 37°c attached to a glass or plastic surface. For growth they require a complex solution of salts, amino acids, vitamins, glucose and unknown factors present in serum. Cells which have multiplied repeatedly may be "passaged", by obtaining in suspension and innoculating into a new culture vessel at a lower cell density.

Primary ("Untransformed") Cell Lines

Cell cultures whose cells "die", ie cease to proliferate, after a number of passages are called primary cell lines. Primary cell lines preserve many of the characteristics of the cells from which they were derived: they have the same number of chromosomes and retain morphological characteristics.

Established Cell Lines

These cells have developed the potential to be subcultured indefinately *in vitro*.

Transformed Cell Lines

(a) Spontaneous transformation: This arises in a primary cell line if, quite suddenly, a few rapidly growing colonies of altered cells appear which quickly outgrow the culture and become the predominant cell type. The cell line has then become an established cell line, ie can be subcultured indefinately.

(b) Viral transformation: Certain tumour viruses can transform some primary culture cells, resulting in the effect described in (a).

Transformed cells have become dedifferentiated (Paul 1970), resulting in morphological and functional changes, and invariably have abnormal chromosome numbers. However, species-specificity is retained (Brand 1962). They have shorter doubling times than their non-transformed counterparts and grow to higher cell densities.

Density Dependent Inhibition of Growth

Transformed cells do not cease to proliferate until the medium in which they have grown is inadequate for nutrition. However, primary cell lines stop growing at a predetermined cell density irrespective of amount of nutrients present. Since many primary cell cultures have been observed to enter a stationary phase about the time they reach confluency, it was originally suggested that the decline in cell growth which occurs might be the result of cell crowding (Puck et al 1956). The phenomenon was described "contact inhibition of growth", by analogy with the observation by Abercrombie and Heaysman (1954) of inhibition of cell movement *in vitro* when cells come into contact.

Direct experimental verification of the contact inhibition of growth theory has not been forthcoming, however and several groups have proposed other terms such as density dependent, post-confluency and cell-cycle inhibition of growth (Stoker and Rubin 1967; Martz and Steinberg 1972; Macieira-Coelho 1967). Although it has been extensively studied, the mechanism of growth rate decline in non-transformed cell cultures has not yet been discovered.

CYCLIC AMP IN CULTURED CELLS

The first reports dealing primarily with cyclic AMP metabolism in cultured cells appeared in 1971 (Gilman and Nirenberg 1971a,b). These reports suggested that cell culture systems were feasible as models for studying cyclic nucleotide metabolism. Being in a "hormonal limbo" cells in culture are not continuously bombarded by signals as are the viable cells of a living multicellular organism.

Basal Cyclic AMP Levels and Cell Growth

Fluctuations of basal intracellular levels of cyclic AMP have been suggested to be involved in the regulation of cell growth in culture. This hypothesis is based on reports that growth rates of cultured cells are inversely proportional to the intracellular concentration of cyclic AMP (Otten et al 1971, 1972; Heidrick and Ryan 1971). Exceptions to this rule (Ney et al 1969; Thomas et al 1973; Burstin et al 1974) cast doubt upon its generality.

Furthermore density dependent inhibition of growth at density restriction in untransformed cells (page 15) has been reported to be associated with a rise in intracellular cyclic AMP. Elevated cyclic AMP has been reported in confluent cultures of various untransformed fibroblast lines, eg human skin fibroblasts (Froehlich and Rachmeler 1972), normal rat kidney fibroblasts (Anderson et al 1973), mouse, monkey, hamster fibroblasts (Rudland et al 1974b). However, work by other authors indicates that it was the depletion of serum factors by the cells rather than "contact inhibition"

or density restriction which signalled the rises in cyclic AMP levels concomitant with the cessation of cell growth observed in cultured fibroblasts. (Sheppard 1972a; Seifert and Paul 1972; Kram et al 1973; Oey et al 1974). Bannai and Sheppard (1974) have reported that while density-dependent cessation of growth in confluent monolayers of 3T3 cells is not correlated with a temporarily synchronous rise in intracellular cyclic AMP, provided the medium is changed daily (Sheppard 1972a)an increase in cyclic AMP levels does occur when the growing cells contact one another.

Seifert and Paul (1972) reported that quiescent (non-dividing) 3T3 cells had two-fold higher cyclic AMP levels than growing 3T3 cells, regardless of whether the cells were in contact with one another or not. However, when serum was added to these quiescent cultures, a decrease in cyclic AMP was observed. Depletion of serum factors leads to cessation of growth in cultured fibroblasts and this is reflected in a rise of intracellular cyclic AMP levels (Otten et al 1972; Seifert and Paul 1972; Kram et al 1973; Oey et al 1974). Alternatively, the administration of fresh serum to serum deprived cultures of fibroblasts results in a rapid decrease in cyclic AMP levels (Sheppard 1972a; Seifert and Paul 1972). This occurs in both normal and transformed fibroblasts.

Deoxyglucose transport was inhibited at cell contact in 3T3 fibroblasts (Bannai and Sheppard 1974). These data suggest that inhibited cellular transport is one of the first biochemical parameters affected by cell contact. In view of

the role of cyclic AMP as a regulator of cellular metabolism as postulated by the second messenger hypothesis, the increase in cyclic AMP may be a critical (though not necessarily primary) event and may serve as a cellular signal which responds to the availability of crucial nutrients. It is thus conceivable that both cell contact and serum factors could affect cyclic AMP levels, possibly through cell transport mechanisms, since many cell membrane parameters would change as a function of normal cell contact eg nutrient transport (Plagemann 1973), distribution of intramembranous particles (Scott et al 1973), plasma membrane enzyme activity (Roth and White 1972), cellular agglutinability (Nicolson and Lacorbière 1973).

Ryan and Heidrick (1974) have likened the elevations of cyclic AMP levels observed in cultured cells under adverse growth conditions, such as serum deprivation, to <u>in vivo</u> situations that result in increased intracellular cyclic AMP levels. Among these were fasting (Selawry et al 1973), circulatory arrest (Wollenberger et al 1969), anoxia and electrical convulsive shock (Goldberg et al 1970).

<u>Effects of Altering Basal Cyclic AMP Levels</u>

Chemical agents which alter intracellular cyclic AMP levels have been shown to affect growth. In 1968 Bürk reported that the addition of methylxanthines to cultures of BHK fibroblasts decreased their growth rate. Methylxanthines have since been shown to increase intracellular cyclic AMP by inhibiting hydrolysis of cyclic AMP by phosphodiesterase (Butcher and Sutherland 1962). Gilman and Nirenberg (1971b) found that PGE_1 raised cyclic AMP in cultured cells and

simultaneously inhibited the rate of cell multiplication. This effect was more dramatic in the presence of theophylline, a methylxanthine. Insulin treatment of fibroblasts has been shown to lead to a transient lowering of cyclic AMP levels, increased DNA synthesis and cell division (Sheppard 1972a,b; Bombik and Burger 1973). Cholera toxin, which activates adenyl cyclase, has been shown to inhibit DNA synthesis (Hollenberg and Cuatrecases 1973). Dibutyrl cyclic AMP, a lipid soluble derivative which is transported into the cell more readily than cyclic AMP (Robinson et al 1971) and is immune to phosphodiesterase digestion (Heersche et al 1971) is known to inhibit the growth of L929 fibroblasts. Concomitant with this is an inhibition of incorporation of ^{3}H-thymidine into DNA (Curtis et al 1973). The above phenomena are extensively catalogued in a review by Ryan and Heidrick (1974).

Conversely, fibroblast growth factor and hydrocortisone have been reported to stimulate growth in quiescent fibroblasts with little or no alterations in cyclic AMP levels (Rudland et al 1974a). Bourne et al (1975) have succeeded in isolating several strains of the S49 mouse lymphosarcoma cell, one of which has no apparent functional adenyl cyclase and hence no endogenous cyclic AMP, and another which has little or no cyclic AMP binding protein and cyclic AMP stimulated protein kinase and is therefore unaffected even by high levels of cyclic AMP. In both of these strains the cell cycle is similar to the parental strains.

While the evidence to date does not implicate cyclic AMP as a regulator of cell growth, it is consistent with alterations in cyclic AMP levels in response to a signal within a model system, for fibroblasts at least, being paralleled by changes in growth rate.

THE PROTEIN BINDING ASSAY

The action of cyclic AMP in all tissues involves binding to a protein (see examples; page 7). Several assay methods based on this principal; "protein binding", or "saturation" assays have been published, eg the method of Brown et al (1971) using a crude adrenal preparation, and the methods of Gilman (1970) and Tovey et al (1974) using a more purified protein kinase from skeletal muscle.

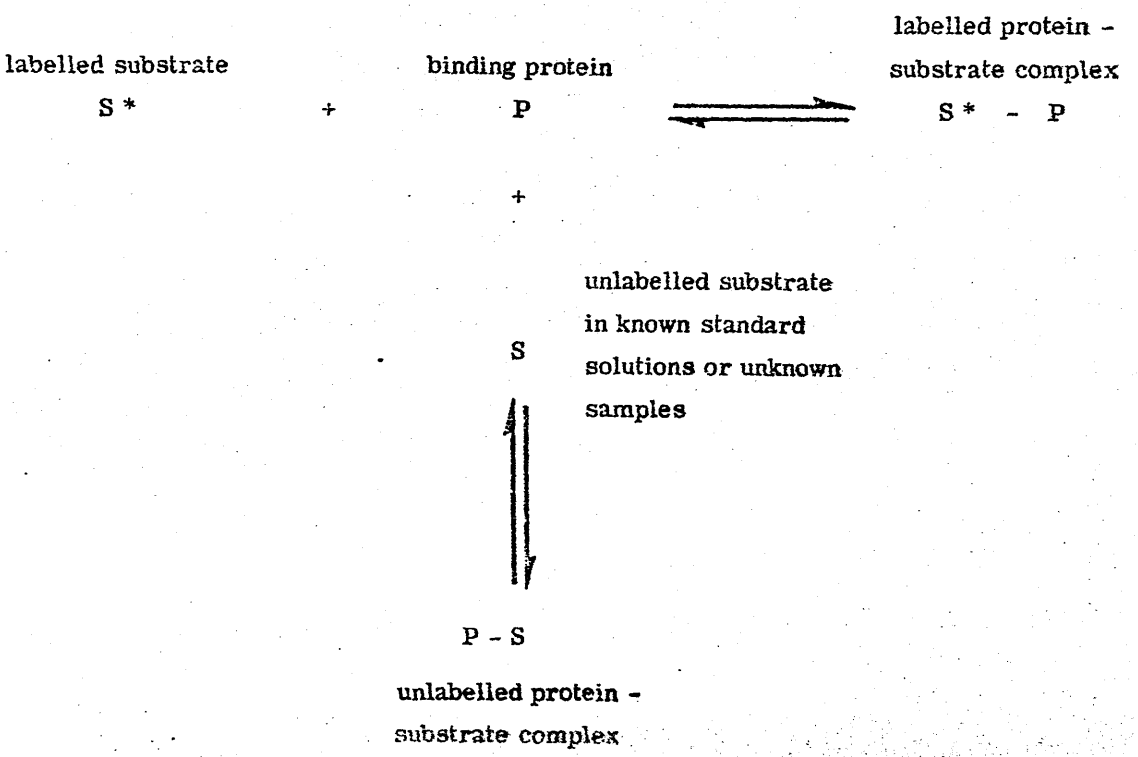
Principal of the Assay

The general principal of the assay is the same as that of radioimmunoassay. A stable substrate S is introduced into a system which contains a constant amount of radioactive substrate S* of high specific activity and its binding protein P. S displaces S* from the substrate binding sites in proportion to its concentration (Fig 5). Removal of unbound substrate is achieved by charcoal adsorption (Brown et al 1971; Tovey et al 1974) or by "millipore" filtration (Gilman 1970). Radioactivity bound gives the degree of competitive inhibition in the unknown and is compared with radioactivity bound in known standard solutions.

Specificity

Validity of results is dependent upon the identical behaviour of standards and unknown, ie the binding protein must be "specific" for cyclic AMP. Specificity is defined as the extent of freedom from interference by substances other than the one intended to be measured (Rees Midgley et al 1969). Lack of specificity in protein binding assays

FIGURE 5



Principal of the protein binding assay.

may primarily arise in three ways (Ekins and Newman 1970):

(a) by competition of another compound for the reaction sites on P

(b) by the influence of an extraneous compound on binding between S and P

(c) by a decrease in efficiency of separation of free and bound fractions.

(a) Competitive Interference

Techniques involving natural binding proteins exploit the almost unique chemical specificity which characterises many biochemical reactions. Consequently, protein binding assays for cyclic AMP have been shown to be free from competitive interference by other nucleotides (eg ATP, cyclic GMP) at their respective physiological concentrations (Gilman 1970; Brown et al 1971; Tovey et al 1974).

(b) Non-Competitive Interference

Of much greater concern are sources of non-specific interference by compounds present in cell and tissue extracts, eg salts of an incubation medium (Bronstrom and Kon 1974), traces of acid used as protein denaturant (Albano et al 1974; Arner et al 1975). Furthermore, different saturation assay methods may have associated with them different non-specific effects, depending on the origin of the binding protein, its degree of purity (Gilman 1970; Walton and Garren 1970), as well as the tissues used for assay.

(c) Decrease in Efficiency at Separation

Differences in protein concentrations have been reported to show marked effects on the characteristics of adsorbants

such as charcoal (Ekins 1970). Divalent cations are known to affect millipore filtration techniques (Albano et al 1974).

Accuracy and Precision

The accuracy of any quantitative estimation is the closeness with which the result approaches the "true" value. Precision may be judged from the statistical scatter about a mean of replicate measurements.

Sensitivity

This property is expressed as the smallest concentration of sample which can be accurately measured by the assay.

On initiation of any assay method, primary investigations must be carried out to optimise assay specificity, accuracy precision and sensitivity.

AIMS OF THE PROJECT

1 To grow cultured cells in concentrations of ethanol similar to those found in the body. L929 cells (Sanford et al 1948), a transformed cell line derived from mouse fibroblasts (Earle 1943) were used. Grown as a monolayer, they provided consistency of experimental conditions, free from hormonal and neurologic influence. Ethanol was added to the cells in concentrations similar to those found in the blood of patients admitted intoxicated to the Western Infirmary.

2 To measure growth parameters in the form of cell counts, DNA, RNA and protein under these conditions.

3 To measure cyclic AMP content in such cells and analyse any relationship to changes found under the influence of ethanol. Intracellular cyclic AMP measurements were obtained using the protein binding assay of Tovey et al (1974). The utilisation of the assay required investigations of specificity, sensitivity, accuracy and precision. Results of these investigations relative to the main theme have therefore been included.

METHODS

PART I

MONOLAYER CULTURE OF L-CELLS

L929 cells were purchased at fortnightly intervals from Gibco-Biocult Ltd., Renfrew, Scotland as a freshly trypsinised suspension containing approximately 1 x 10^6 cells/ml. For experiments, the cells were subcultured on to petri dishes as monolayers, under aseptic conditions. Growth medium consisted of Medium 199 (Morgan et al, 1950) with Hanks' Balanced Salt Solution (Hanks and Wallace, 1949), foetal calf serum (10%) and glutamine (2mM). Hepes buffer (25mM) was also included to maintain a constant pH of 7.4 in the medium. Medium 199 was obtained commercially as were the other added constituents. The constituents of Medium 199 and Hanks' BSS are shown in Tables IV and V.

During the fortnightly intervals, stock cultures were maintained of cell monolayers in 70cm^2 or 120cm^2 glass bottles. For subculturing, cells were removed from the bottle by rinsing the cell layer with approximately 20ml trypsin (0.25%) in Ca and Mg free Hanks' BSS, which was immediately poured off. After 5 mins incubation at room temperature, during which the cells became detached from the glass, 20ml growth medium (with serum added) was added to inhibit further activity of the trypsin. The cells were universally dispersed throughout this medium and large clumps of cells separated by successively aspirating the suspension using a sterile 10ml pipette with cotton wool plug.

TABLE IV

Constituents of Medium 199

Amino Acids

	Conc mg/L		Conc mg/L
DL-Alpha-Alanine	50.00	DL-Isoleucine	40.00
L-Arginine HCl	70.00	DL-Leucine	120.00
DL-Aspartic acid	60.00	L-Lysine monohydrochloride	70.00
L-Cysteine HCl	0.10	DL-Methionine	30.00
L-Cystine	20.00	DL-Phenylalanine	50.00
DL-Glutamic acid monohydrate	150.00	L-Proline	40.00
L-Glutamine	100.00	DL-Serine	50.00
Glycine	50.00	DL-Threonine	60.00
L-Histidine HCl	20.00	DL-Tryptophan	20.00
L-Hydroxyproline	10.00	DL-Valine	50.00

Vitamins

	Conc mg/L		Conc mg/L
Ascorbic acid	0.05	Menadione	0.01
d-Biotin	0.01	Niacin	0.025
Calciferol	0.10	Niacinamide	0.025
Ca pantothenate	0.01	Para-Aminobenzoic acid	0.05
Choline Cl	0.50	Pyridoxal HCl	0.025
Disodium alpha tocopherol phosphate	0.01	Pyridoxine HCl	0.025
Folic acid	0.01	Riboflavin	0.025
Glutathione	0.05	Thiamine HCl	0.01
i-Inositol	0.05	Vitamin A	0.10

TABLE IV cont'd

Nucleic Acid Constituents and Accessory Growth Factors

	Conc mg/L		Conc mg/L
Adenine sulphate	10.00	Hypoxanthine	0.30
Adenosinetriphosphate (di-Na salt)	1.00	Ribose	0.50
Adenylic acid	0.20	Sodium acetate	50.00
Cholesterol	0.20	Thymine	0.30
Deoxyribose	0.50	Tween 90*	20.00
Ferric nitrate	0.10	Uracil	0.30
Guanine HCl	0.30	Xanthine	0.30

*Trademark of Atlas Powder Co

TABLE V

Constituents of Hanks' Balanced Salt Solution

	Conc mg/L
NaCl	8000.0
KCl	400.0
$MgSO_4.7H_2O$	200.0
$Na_2HPO_4.2H_2O$	60.0
KH_2PO_4	60.0
Glucose	1000.0
Phenol red	20.0
$CaCl_2$ (anhyd.)	140.0
$NaHCO_3$	350.0

Cell Counting

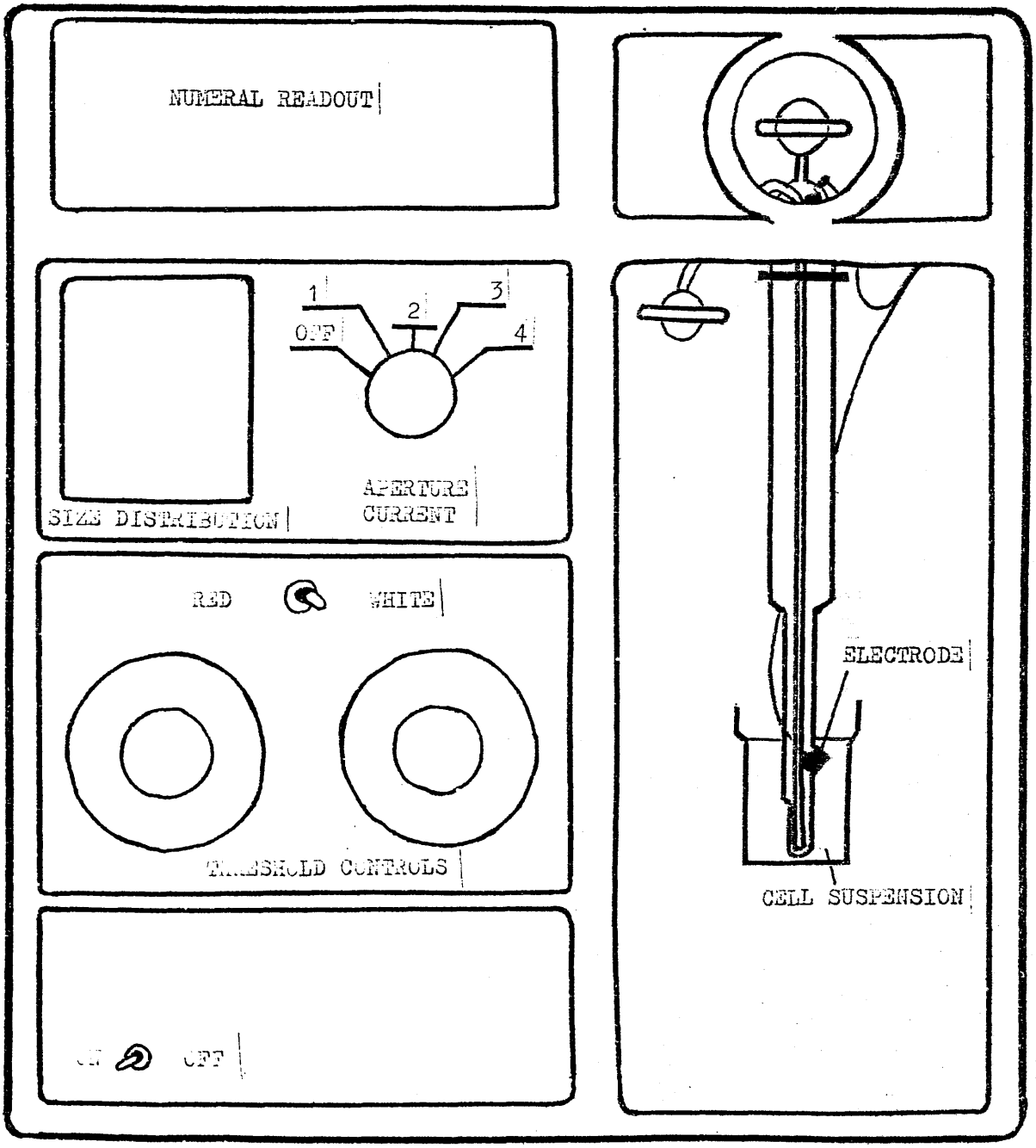
A diluted sample of the trypsinised cell suspension was counted using a Coulter Counter, Model D (Fig 6). A 40 µl aliquot of the cell suspension was added to 20ml normal saline (0.15M NaCl) in a Coulter "accuvette" container using a blow out pipette. This was inverted then inserted into the machine. Cell counts registered were cells/mm^3 original cell suspension. A background count consisting of 40 µl medium in 20ml saline was subtracted from all counts. Total background count was never greater than 5% of total cell counts.

The settings employed on the Coulter Counter were as for white blood cell counting, viz an aperture current setting of 3 using a 100µ orifice. The threshold was adjusted to a value of 20, found to be the point where all cells were counted and background noise was minimal. The counting profile of L-cells is shown in Fig 7A. Counts obtained were cross-checked by counting random plates by hand under the microscope using a haemocytometer chamber, using the technique employed for white blood cell estimations. Counts obtained by haemocytometer versus counts obtained on the Coulter Counter resulted in a linear correlation ($r = 0.91$ for range $1\text{-}10 \times 10^4$ cells/mm^3; Fig 7b)

Cell Viability

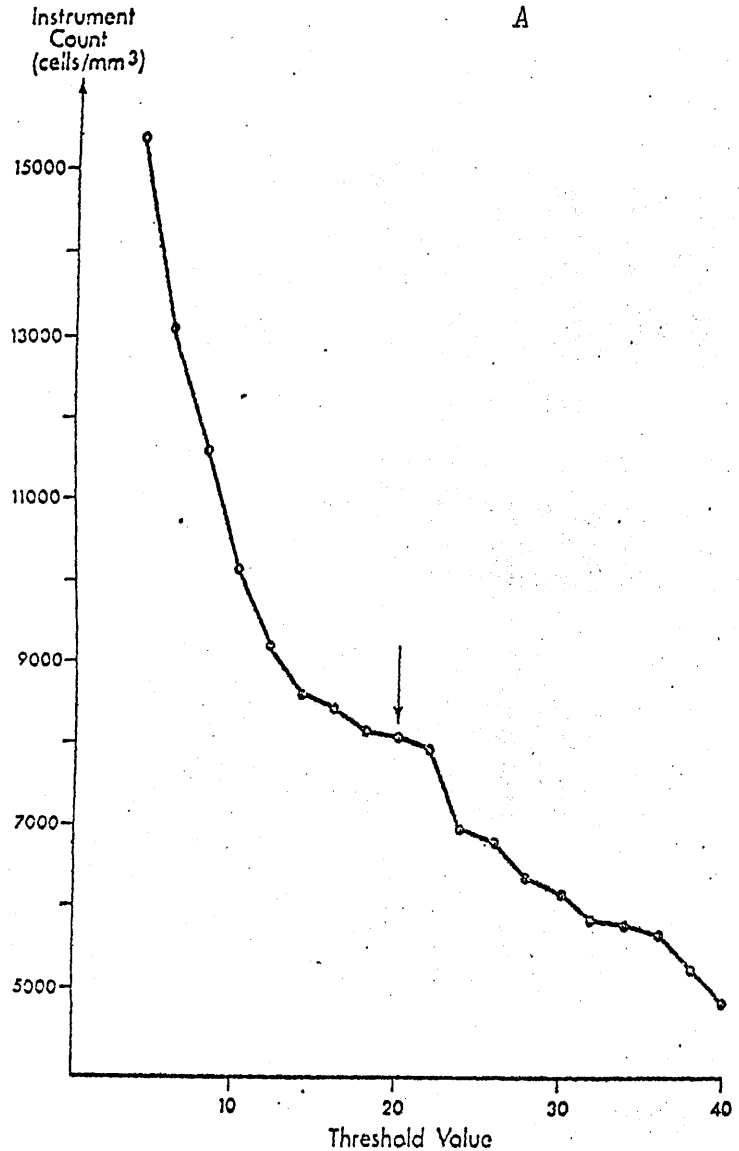
Estimates of cell viability were carried out on both the commercially obtained cells as well as those subcultured in the laboratory. Trypan blue (4%; 0.1ml) in normal saline was added to 0.9ml cell suspension containing approximately 10^6 cells/ml. The mixture was aspirated using a pasteur pipette

FIGURE 6

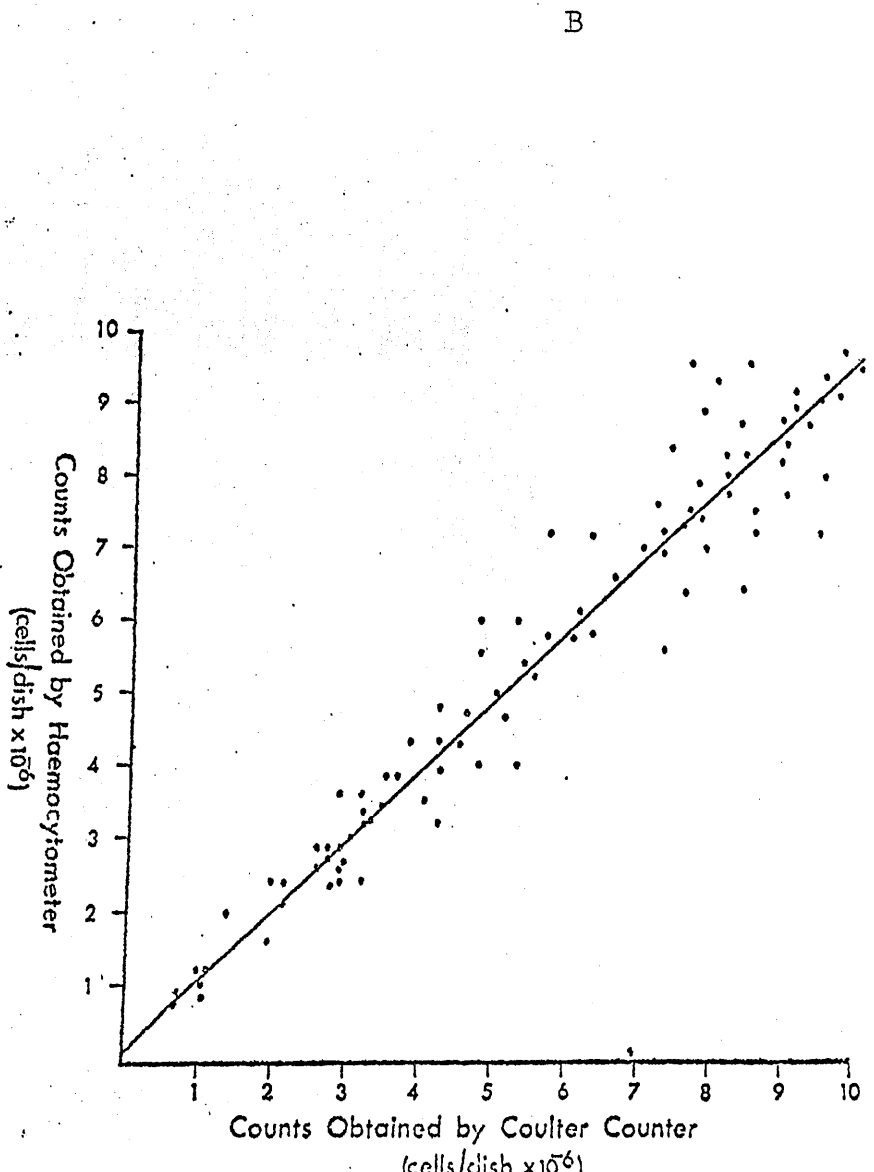


The Coulter Counter

FIGURE 7



Counting profile of L929 cells on the Model D Coulter Counter using aperture current setting 3 and 100 u orifice.



Correlation between counts obtained by haemocytometer and counts obtained on the Coulter Counter.

and a drop of this stained suspension placed in a haemocytometer chamber and examined using an inverted microscope. After 10 mins the total number of cells and the number of dead (staining) ones were counted in several fields. Visibility, calculated by the formula

$$\frac{\text{total cells - dead cells}}{\text{total cells}} \times 100\%$$

was always between 95% and 100% in cells used for experiment.

Subculture Techniques

In preliminary experiments, the initial plating density of the cells was found to be critical to subsequent occurence, timing and rate of cell multiplication. Initially, cells were aseptically diluted in 500 ml freshly prepared growth medium as described above, to give a final concentration of 2×10^5 cells/ml. Sterile pipettes were used to deliver 10 ml aliquots to plastic petri dishes (10 cm diameter). The dishes were incubated at 37°c in an air atmosphere for at least 48 hrs before initiation of experimental conditions. Cells were used for experiment when the cell density reached $3.5 \pm 0.4 \times 10^6$ cells per plate.

In a later series of experiments cells were plated at a density of 3.5×10^6 cells per dish. These cells were used for experiment when the cell density reached $4.5 \pm 0.4 \times 10^6$ cells per dish.

Experimental Media

The concentrations of ethanol (Absolute Alcohol; 99.9% in the media used in experiments was regularly checked by Gas Liquid Chromatography (glc) determinations (Walls and

Brownlie, 1970). A Pye Series 104 gas liquid chromatograph with flame ionisation detector was employed. Nitrogen was used as carrier gas, Perkin Elmer Carbowax column packing and an oven temperature of 88°c. An accurate dilution was made with the ethanol containing medium and an internal standard solution of n-propanol using a Griffin & George 219 Haemoglobin-type diluspence. An ethanol standard solution, the concentration of which is accurately known (±0.01mM) was similarly treated. 1 µl aliquots of each were injected successively into the column using an SGE syringe (1 µl capacity).

The alcohol in each sample immediately vapourises upon injection (column temperature 88°c). The ethanol and propanol in each sample are carried through the column at different rates because they are adsorbed to different extents by the column packing. Hence they emerge at different times. The flame ionisation detector is sensitive to changes in the composition of the issuing gas and feeds electrical impulses according to these into a recorder, giving rise to a peak on a paper strip. The area of each peak is directly proportional to the concentration of alcohol in the sample. The concentration of ethanol in the unknown sample is calculated as follows:

$$\frac{\text{Ethanol conc of unknown}}{\text{Ethanol conc of standard}} = \frac{E/_P \text{ unknown}}{E/_P \text{ standard}}$$

where

E = area of peak for ethanol standard

P = area of peak for n-propanol standard

The concentration of ethanol in the medium used for experiments never varied by more than 0.5mM/L between experiments. Furthermore the purity of the ethanol was regularly examined

using glc and was always found to give 1 peak.

Experimental Procedure

Growth medium containing ethanol at concentrations of 21mM (0.13%) and 42mM (0.25%) was made up by adding 0.75ml and 1.5ml respectively of absolute ethanol to 600ml medium.

At the start of the experiment, dishes were divided into three groups, with 20-25 dishes per group. Medium was poured off and replaced with 10ml medium with ethanol concentrations of zero (control group), 21mM and 42mM (test groups). Further medium changes were made daily for four consecutive days.

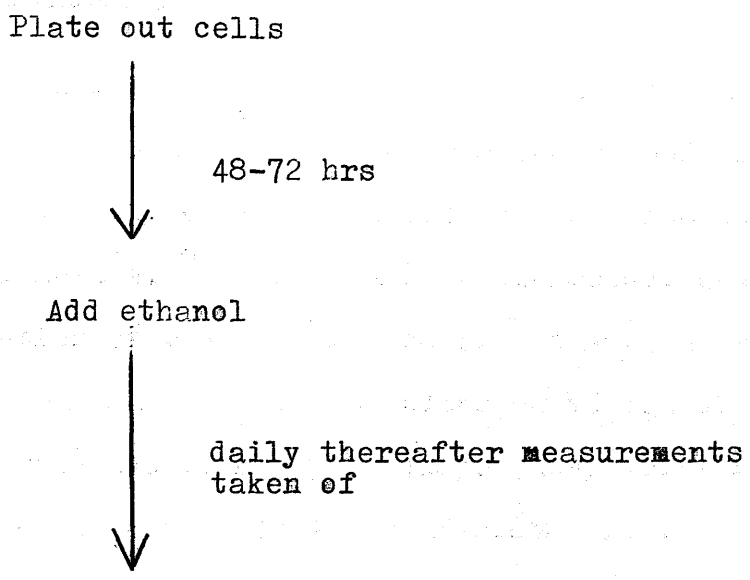
Estimation of Cell Population

At 24hr intervals from cell plating until the end of each experiment, two randomly selected dishes from each of control and test groups were removed and cell counts per dish taken in duplicate. Medium was poured off the plate and residual medium removed using a pasteur pipette. The cell monolayer was then resuspended in 1ml saline using a glass rod with rubber stopper and the cells dispersed by aspiration using a pasteur pipette. The cells were counted by Coulter Counter as described above (page 26). Variation in cell number between individual plates throughout any experiment was never more than 8%.

Growth Parameters: DNA, RNA, Protein and Cyclic AMP

Daily measurements were taken of intracellular DNA, RNA, protein and cyclic AMP; to be further described in Parts II and III of this section. Fig 8 summarises the experimental procedure.

FIGURE 8



(1) Cell population per dish

(2) Intracellular cyclic AMP

(3) Intracellular protein, RNA, DNA

Experimental procedure.

PART II

THE CYCLIC AMP ASSAY

Assay for cyclic AMP was carried out using a commercially obtained protein-binding assay kit, which uses the method of Tovey et al (1974). The kit contains binding protein isolated from bovine skeletal muscle, ^{3}H-cyclic AMP (29Ci/mmole), charcoal adsorbant for separation of bound from free cyclic AMP and standard cyclic AMP for construction of a standard curve containing 0 to 16 picomoles (pmol) cyclic AMP per assay. Assay buffer, included in all of the reagents, consists of 0.05M Tris, 4mM EDTA, pH 7.5 (Tris/EDTA buffer). The reagents are supplied in freeze-dried form to be reconstituted with distilled water prior to use.

Extraction of Cyclic AMP: Preliminary Investigations

There is no published evidence to date of the method of Tovey et al (1974) being applied to measurement of cyclic AMP in tissue culture systems. With the knowledge (page 21) that non-specific effects resulting from methodology can be troublesome in protein binding assays, a comparison was made between various agents used in practice to inactivate cyclic AMP metabolism in tissue culture systems. Precision of replicate results for any one extraction reagent was taken as an indication of error involved in extraction method.

The extraction reagents investigated were:

(a) Tris/EDTA buffer

(b) 0.1N Hydrochloric acid (HCl)

(c) 0.5M Perchloric acid (PCA)

(d) 5% w/v Trichloroacetic acid (TCA)

After harvesting the cells for assay of cyclic AMP, the medium was removed by aspiration and the appropriate extraction reagent (3ml, ice-cold) immediately added to the cell sheet. Each extraction reagent contained 12.5nCi/ml ^{3}H-cyclic AMP (0.02 pmol/assay) to monitor cyclic AMP recovery. The resulting lysed cell sheet was removed using a glass rod with rubber stopper. For dishes containing less than 6 x 10^{6} cells, three dishes were used and the cell extract rinse transferred to the second and third dish. For dishes containing more than 6 x 10^{6} cells, two dishes were used.

The cell extract was then homogenised by hand using a glass homogeniser, to release all intracellular cyclic AMP into solution. Protein, together with other cell debris was removed by centrifugation (3000g, 15 mins, 4^{o}c) and the dishes rinsed again with the supernatant fraction. Centrifugation was repeated and 50 µl of the supernatant was removed for liquid scintillation counting for calculation of recovery. At this point care had to be taken to ensure that the pH of supernatants was returned to the pH of assay (7.5) to allow maximal efficiency of binding during the assay.

Preparation of Supernatants for Assay of Cyclic AMP

(a) Supernatants obtained by homogenisation of cells in Tris/EDTA buffer were used directly in the assay.

(b) HC1-containing supernatants were neutralised (pH 7.5) using saturated potassium hydroxide (KOH), lyophylised and resuspended in Tris/EDTA buffer.

(c) Supernatants resulting from PCA extraction were neutralised (pH 7.5) using saturated KOH and the resulting precipitate of potassium perchlorate ($KC1O_4$) removed by centrifugation.

The supernatant was lyophylised and resuspended in Tris/EDTA buffer.

(d) TCA containing supernatants were extracted six times with 10 ml water saturated ether. TCA is largely removed in the ether phase, whereas the cyclic AMP remains in the aqueous phase. The ether used was saturated with water to prevent a decrease in the sample volume. Final traces of ether were removed by placing the sample in a boiling water bath for 3 mins, a procedure which results in negligible hydrolysis of the cyclic AMP, cyclic AMP having a half-life of hydrolysis of 55 mins in 1N acid at 92°c (Lipkin et al 1959).

The acidic pH of the cell extracts suggested that traces of TCA remained. Extracts were either lyophylised and resuspended in assay buffer (final pH 7.5), or further purified by ion-exchange chromatography using a Dowex cation exchange resin column, (AG-50W x 8, 100-200 mesh, H^+ form). For each sample to be purified, a column (0.5 x 7 cm) was prepared using a disposable pasteur pipette into which had been inserted a small cotton wool plug. A slurry of resin was prepared by stirring 75g of the resin with 100 ml distilled water and 10 ml of this suspension was transferred to the column. Each column was washed with 5 ml 1NHC1, then repeatedly with distilled water until the pH of eluate returned to that of distilled water. Then the ether-removed TCA extract was added to the column.

Cyclic AMP was eluted using distilled water, in the eighth to tenth ml timed from the addition of sample to the resin. Its elution profile was monitored using the ^3H-cyclic AMP tracer.

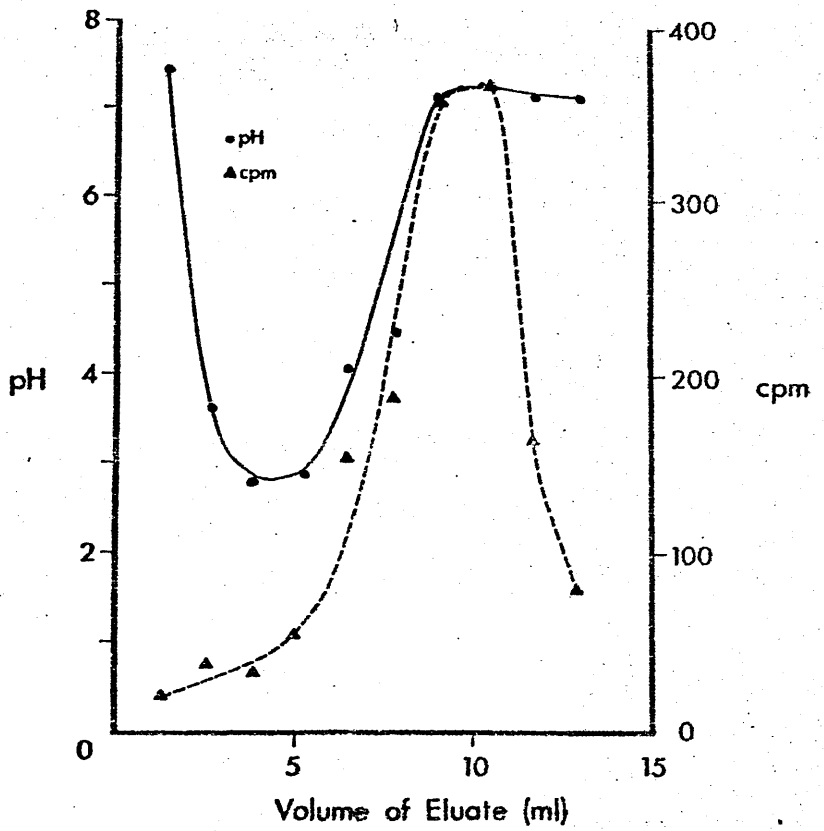
TCA remaining after ether extraction emerged immediately before the cyclic AMP (second to eighth ml) and cyclic AMP eluted at the pH of distilled water. Fig 9 shows the elution profiles of cyclic AMP and TCA. Fractions corresponding to the "peak" of cyclic AMP elution, as monitored by the tracer, were pooled. The ion-exchange procedure caused a x4 - x3 dilution of the cyclic AMP in comparison to its concentration in the original TCA extract. Thus the pooled fractions were lyophylised and resuspended in ¼ their volume of Tris/EDTA buffer. Average recovery of cyclic AMP from the resin was 75%; never lower than 70% (tending to vary slightly with volume of sample).

The Assay Method

For each extraction procedure, 50 µl of the redissolved extract was removed for liquid scintillation counting for calculation of recovery and 50 µl in duplicate for assay of cyclic AMP. Methodology is summarised in Figs 10 and 11.

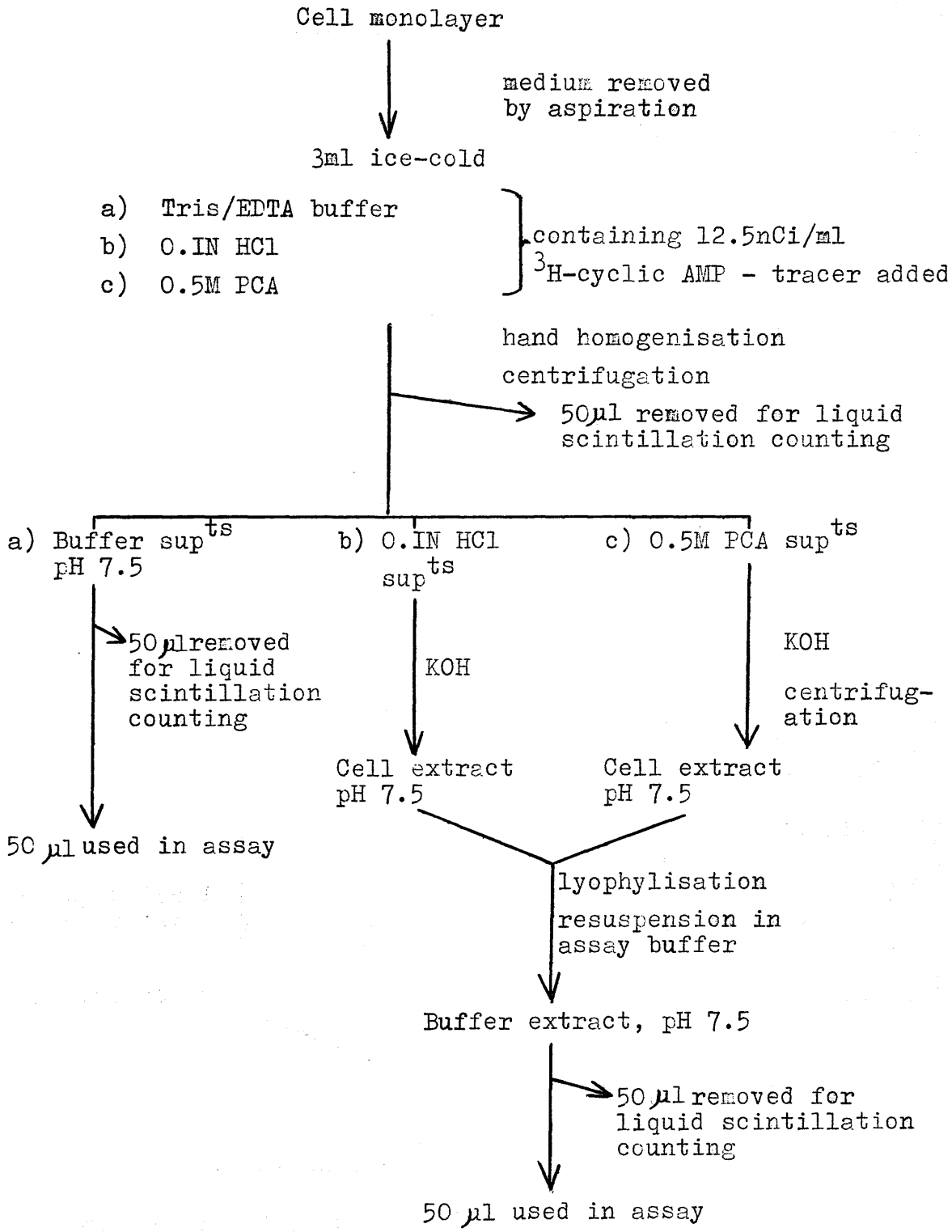
The standard binding reaction was performed rigidly adhering to the kit instructions. Radioactivity was estimated by liquid scintillation counting. Blank values, obtained by measuring the radioactivity in the supernatant after charcoal adsorption in the absence of binding protein, were subtracted from all results. Results were expressed in terms of Co (cpm in the absence of unlabelled cyclic AMP or "zero" and divided by Cx (cpm in the presence of unlabelled cyclic AMP). In each case the measurements were referred to those of standard cyclic AMP diluted in Tris/EDTA buffer. A linear calibration curve for $^{Co}/_{Cx}$ against varying cyclic AMP concentrat-

FIGURE 9



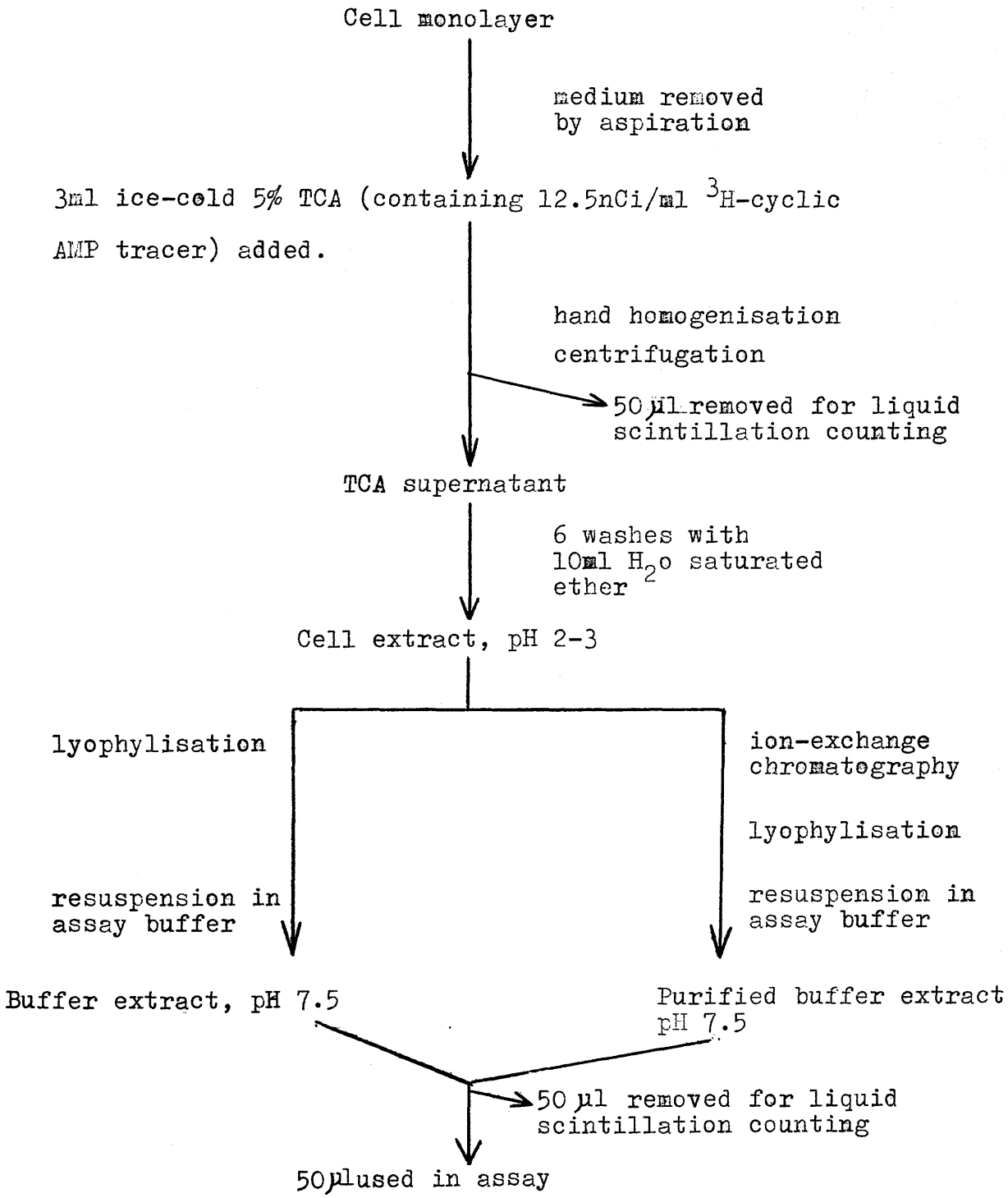
Elution profiles of cyclic AMP, as shown by cpm of the ^{3}H cyclic AMP tracer and TCA, as shown by pH, from the Dowex 50 resin. For details, see text.

FIGURE 10



Extraction of intracellular cyclic AMP: procedures used for agents Tris/EDTA buffer; hydrochloric acid; perchloric acid.

FIGURE 11



Extraction of intracellular cyclic AMP: procedure used for agent trichloroacetic acid.

ions could be obtained for each assay. (Figs 12 to 15).

Table VI compares apparent cyclic AMP measurements obtained using each of the above methods on dishes grown under identical conditions and containing identical cell populations, within experimental error. In assay 1, cells were harvested at a population density of 3.5×10^6 cells/dish. In assay 2, cells were harvested at a higher population density of 6.2×10^6 cells/dish. In both cases, in this experiment, 3 dishes were used for each extraction agent.

Precision of results was indicated by coefficient of variation between quadruple measurements. This coefficient of variation was accepted as representing the error involved in extraction method. Within-assay coefficients of variation for quadruple standards within the range 0.5 - 2 pmol cyclic AMP/assay for assays 1 and 2 were 9.3 and 6.2 respectively. Only Tris/EDTA buffer extraction and TCA extraction including ion-exchange chromatography purification gave results within this degree of precision. (coefficients of variation $\leqslant 9.6$). Variations in apparent cyclic AMP results obtained were high between and within extraction methods used. PCA and TCA extracts which had not been processed by ion-exchange chromatography showed relatively high degrees of non-specificity (coefficients of variation $\geqslant 12.7$), indicating interference from extraction method.

Extraction of Cyclic AMP: Further Investigations

(a) Tris/EDTA Buffer and (b) 0.1N Hydrochloric Acid Extraction

These reagents had been recommended by the suppliers of the kit for use in extracting cyclic AMP from tissue culture

TABLE VI

Within-Assay Precision for Extraction Methods Investigated

Assay 1: 3.5×10^6 cells/dish

Extraction method	Cells/assay $\times 10^6$ (corrected for recovery)	Apparent pmol cyclic AMP per assay $\pm$ SD(n=4)	Coefficient of variation
Tris/EDTA buffer	0.18	0.42 ± 0.04	9.2
0.1N HCl	0.22	0.50 ± 0.05	9.6
0.5M PCA	0.21	1.70 ± 0.31	18.2
5% TCA			
a) ether removal	0.27	1.26 ± 0.16	12.7
b) ether removal plus ion-exchange chromatography	0.12	0.95 ± 0.03	2.9
Standard cyclic AMP @ 2 pmol/assay	-	2.08 ± 0.19	9.3

Assay 2: 6.2×10^6 cells/dish

Extraction method	Cells/assay $\times 10^6$ (corrected for recovery)	Apparent pmol cyclic AMP per assay $\pm$ SD(n=4)	Coefficient of variation
Tris/EDTA buffer	0.31	0.97 ± 0.07	7.0
0.1N HCl	0.30	0.76 ± 0.07	9.3
0.5M PCA	0.25	2.17 ± 0.31	14.3
5% TCA			
a) ether removal	0.31	1.54 ± 0.96	62.3
b) ether removal plus ion-exchange chromatography	0.35	1.35 ± 0.12	9.0
Standard cyclic AMP @ 1.5 pmol/assay		1.56 ± 0.10	6.2

cells (personal communication). The advantages stated were high recovery, simplicity of methodology as well as freedom from interference resulting from extraction reagent.

(a) EDTA inactivates cyclic AMP metabolism by chelating Mg^{2+}, necessary for the activity of both adenyl cyclase (Sutherland et al, 1962) and phosphodiesterase (Butcher and Sutherland, 1962). EDTA has been reported as potently inhibiting plasma phosphodiesterase (Tovey et al 1974) as well as phosphodiesterase in broken cell preparations (Cheung, 1970). However there is no evidence reported suggesting that this reagent acts instantaneously in whole cell preparations.

(b) O.IN HCl, inactivating metabolism by virtue of its acidity, is neutralised to KCl. The assay has been shown to be free from interference in the presence of salts at concentrations as high as 1 Molar (Tovey et al, 1974). However it is reported that this reagent is inadequate as an inactivator of cyclic AMP metabolism in membrane fragments of cerebral cortex (Weller et al, 1972).

Damage to the cells was not apparent immediately upon addition of either Tris/EDTA buffer or O.IN HCl as examined using the light microscope. Although precision was high within anyone assay for each of these extraction reagents (coefficients of variation were of the same magnitude as that obtained for the standard at 2 pmol/assay; Table VI), results could not be reproduced from experiment to experiment with this degree of precision. Inter-assay coefficients of

variation for replicate experiments using these reagents were often twice those obtained for standards (results not shown). Thus insufficient inactivation of metabolism leading to high scatter of results between replicate experiments, which could not be attributed to inter-assay variation, was evident. A further experiment was carried out to decide if results obtained for measured cyclic AMP were related to the time between addition of inhibitor and homogenisation of the cells. It was found (Table VII) that apparent measured cyclic AMP varied as time lapse between addition of buffer or 0.1N HCl and homogenisation varied. The rate limiting step in inactivation of cyclic AMP metabolism using these reagents was therefore presumed to be homogenisation of the cells.

Buffer and 0.1N HCl extraction methods were rejected as being insufficiently rapid in inactivating metabolism.

(c) Perchlorate (PCA) Extraction

This reagent is in practice widely used as an extraction reagent for cyclic AMP (Weinryb, 1972; Greengard and Robinson, 1972). Its outstanding advantage is its subsequent ease of removal via a neutralisation reaction resulting in a precipitate of $KClO_4$. However in measuring nanomolar quantities of cyclic AMP it seemed feasible that equilibrium concentrations of perchlorate present at neutralisation might interfere in the cyclic AMP assay.

Preliminary investigations showed that apparent pmol cyclic AMP/assay in cells harvested with perchlorate gave rise to within-assay replicate measurements with high scatter

TABLE VII

Reagent	Time interval between adding reagent and homogenising cells	Apparent pmol cyclic AMP per assay ±SD (n=4) (Corrected for recovery)
Assay 1:		
Tris/EDTA buffer	immediate	1.21±0.04
	1 min	0.62±0.03
	2 mins	0.93±0.17
	5 mins	0.73±0.08
Assay 2:		
0.1N HCl	immediate	0.59±0.05
	1 min	1.07±0.10
	2 mins	0.66±0.08
	5 mins	0.45±0.02

Variation in intracellular cyclic AMP measurements with time taken to homogenise the cell extracts.

In Assay 1 (Tris/EDTA buffer extraction), cells were harvested at a population density of 3.5×10^6 cells per dish. In Assay 2 (0.1N HCl extraction) cells were harvested at a population density of 5.3×10^6 cells per dish. For each time interval, three dishes were used, as described in the text. The time interval was measured from time of addition of the cell extract rinse to the third dish.

Within-assay coefficients of variation were 27.5 and 36.2 for Assays 1 and 2 respectively.

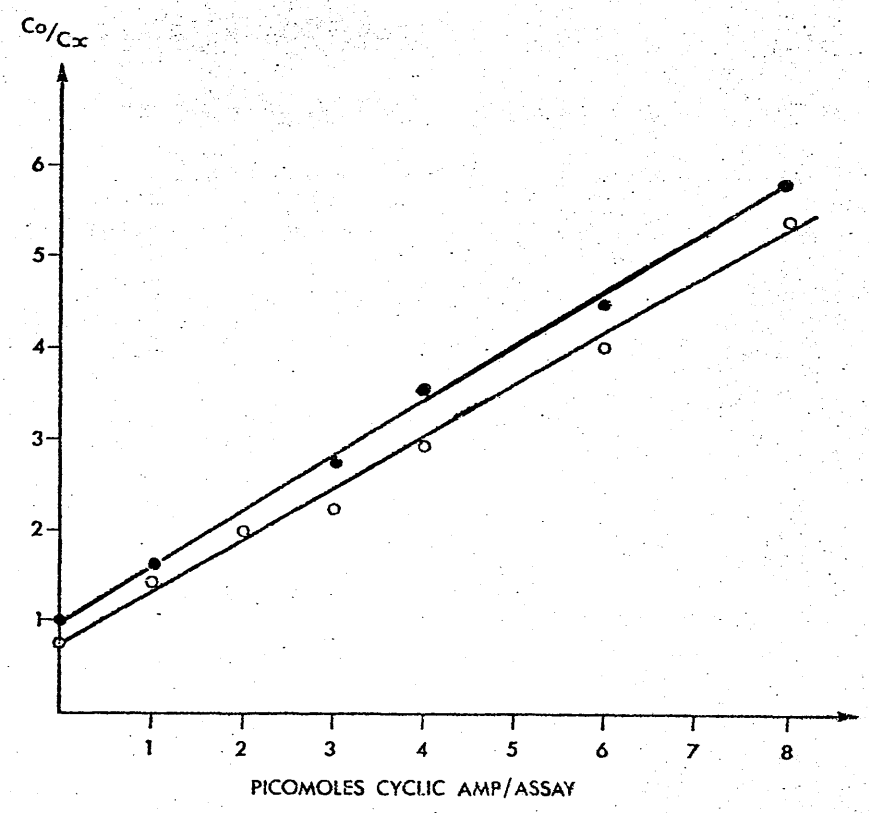
in comparison to standards (coefficient of variation was approximately twice that of standard; Table VI). Thus interference from extraction method was evident. Therefore, preliminary experiments to investigate validity of results were carried out.

(i) Cell Extract Blank

Cell extracts, using perchlorate extraction, were prepared as described above (page 31). Extracts were depleted of cyclic AMP using exogenous cyclic nucleotide phosphodiesterase, as follows: beef heart 3',5'-cyclic nucleotide phosphodiesterase, obtained as a lyophylised powder, was diluted in distilled H_2O to yield 1.7 units/ml (one unit being defined as the amount of enzyme which will convert 1 μ mole of 3',5'-cyclic AMP to 5'-AMP per minute at pH 7.5 at 30°c). This was added to the neutralised (pH 7.5) cell extract after perchlorate extraction, to give a concentration of 17mU/ml. Incubation was carried out at 30°c for 2 hrs and the extract boiled for 3 mins to denature the enzyme.

Crystalline cyclic AMP (monosodium salt) was diluted in this extract to yield the concentrations of standards which comprise the standard curve used, viz 1 to 8 pmol/50 μl. Control samples were prepared consisting of crystalline cyclic AMP dissolved in distilled water. In each case, the standards were lyophylised and resuspended in buffer for assay. The standard assay procedure was then followed, to yield the response curves shown in Fig 12 . The standard curve using a cyclic AMP-depleted cell extract is displaced downwards when

FIGURE 12



Standard curve for standards diluted in H_2O (.) or in cell extracts of 0.5N PCA depleted of cyclic AMP using exogenous phosphodiesterase (O). In each case the standards were lyophylised and resuspended in Tris/EDTA buffer, as described in methods. Control curve :r = 0.99. Cell extract curve :r = 0.92.

compared to the control curve, giving rise to an over-estimate of measured cyclic AMP. Precision and sensitivity have been diminished.

(ii) Reagent Blank

A reagent blank was prepared by neutralising 0.5M PCA and removing the precipitate of $KClO_4$. Standards diluted in this reagent blank, lyophylised and resuspended in Tris/EDTA buffer for assay gave rise to the standard curve shown in Fig 13 . It is apparent from Fig 13 that the diminution in sensitivity originated from the perchlorate, the precision being improved (correlation coefficient increased) but the curve deflection unaltered. These findings qualitatively agree with Albano et al (1974) who used a binding protein from the cortices of adrenal glands.

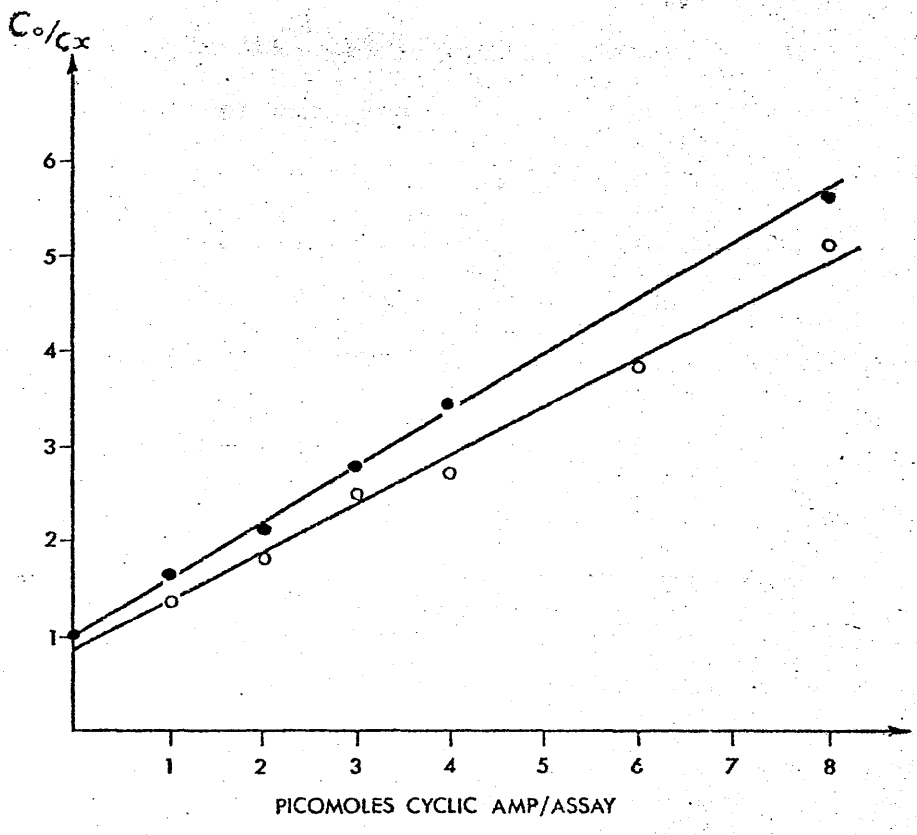
For these reasons, it seemed prudent to abandon perchlorate as extraction reagent.

(d) Trichloroacetate (TCA) Extraction

This reagent is by far the most widely used extraction reagent for cyclic AMP in tissue culture (Weinryb, 1972; Greengard and Robinson, 1972).

Table VI indicates that results obtained for apparent pmol cyclic AMP/assay using TCA as extraction reagent gave rise to unacceptable within-assay variations for replicates. Further purification of the ether-removed TCA extract by ion-exchange chromatography gave rise to results with acceptable within-assay precision, compared to standards diluted in buffer (coefficients of variation $\leq$ that obtained for standard at 2 pmol/assay; Table VI).

FIGURE 13



Standard curve for standards diluted in H_2O (.) or in neutralised 0.5N PCA reagent blank. (O). In each case the standards were lyophylised and resuspended in buffer, as described in methods. Control curve :r = 0.99. PCA reagent blank curve :r = 0.96.

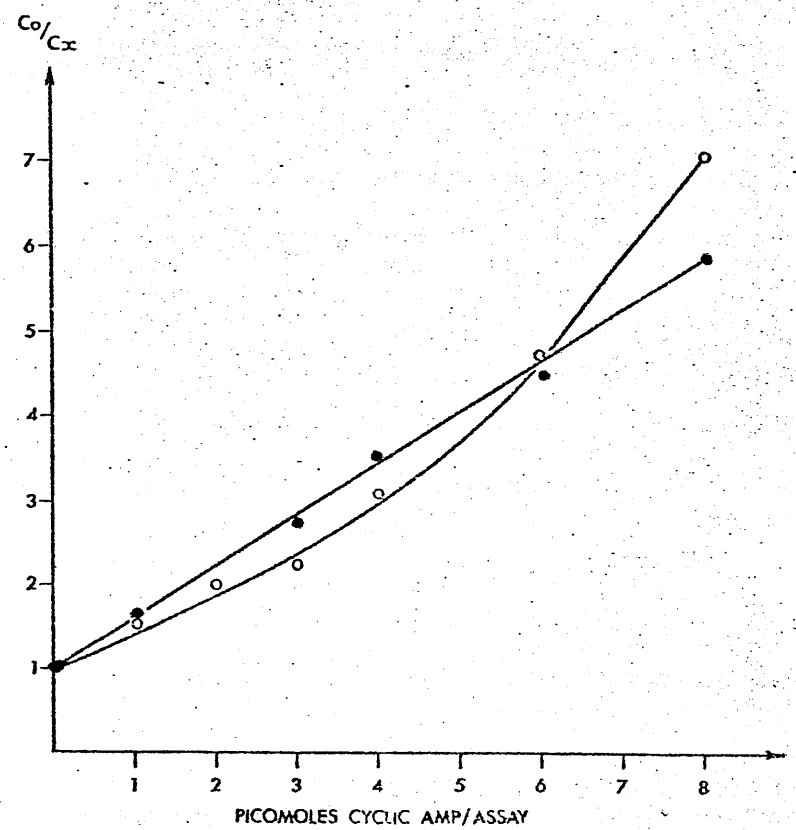
The mechanical method of ether extraction for removal of trichloroacetate from aqueous solution is well established (Greengard and Robinson, 1972). This method has the advantage that its efficiency of removal from the sample may be monitored by pH measurements. The removal methods described above (page 32), are particularly applicable to an aquecus sample: ether is immiscible with water and can be completely removed by evaporation; Dowex 50 columns (Schultz et al, 1975) are especially useful if the samples contain large amounts of electrolytes (tissue constituents, salts of an incubation medium, acid used for extraction), since the sample can be directly applied.

(i) Reagent Blank of TCA

Since it was established that the interference resulting from PCA extraction methods mainly due to the reagent, interference (if any) from a reagent blank of TCA was primarily investigated. This was prepared by washing 3ml 5% TCA six times with 10ml water saturated ether. Crystalline cyclic AMP was dissolved and diluted in this reagent blank to yield the standards which comprise the standard curve. Again, control samples were prepared consisting of crystalline cyclic AMP dissolved in distilled water. In each case the standards were lyophylised and resuspended in assay buffer and the standard assay procedure followed.

Fig 14 shows that when 5% TCA was used as the extraction reagent a different effect than that of the PCA reagent blank was obtained. The change in standard curve is related in a more complex fashion to the cyclic AMP in the assay. Samples

FIGURE 14



Standard curve for standards diluted in H_2O (.) or in ether-removed 5%TCA reagent blank (0).

containing less than six pmol cyclic AMP/assay would produce a falsely high measured cyclic AMP if referred directly to the standard curve, a result qualitatively paralleled by Albano et al (1974). Non-specific interference from ether-extracted lyophylised TCA extracts of tissue have been reported by other investigators (Arner and Östman 1975, Sanborn et al, 1973). Each of these authors advocated the use of a lyophylisate of ether-washed TCA in the assay buffer to produce more valid estimations of measured cyclic AMP. Such an approach assumes interference originating only from the reagent, and would give rise to a non-linear standard plot.

Dowex ion-exchange chromatography as described above, included a further step in purification of the reagent blank completely removed the source of this effect, yielding a standard curve identical to control within experimental limits (not shown). I have concluded that traces of TCA remaining after ether extraction had contributed to the imprecision of results obtained in Table VI.

(ii) Cell Extract Blank of TCA

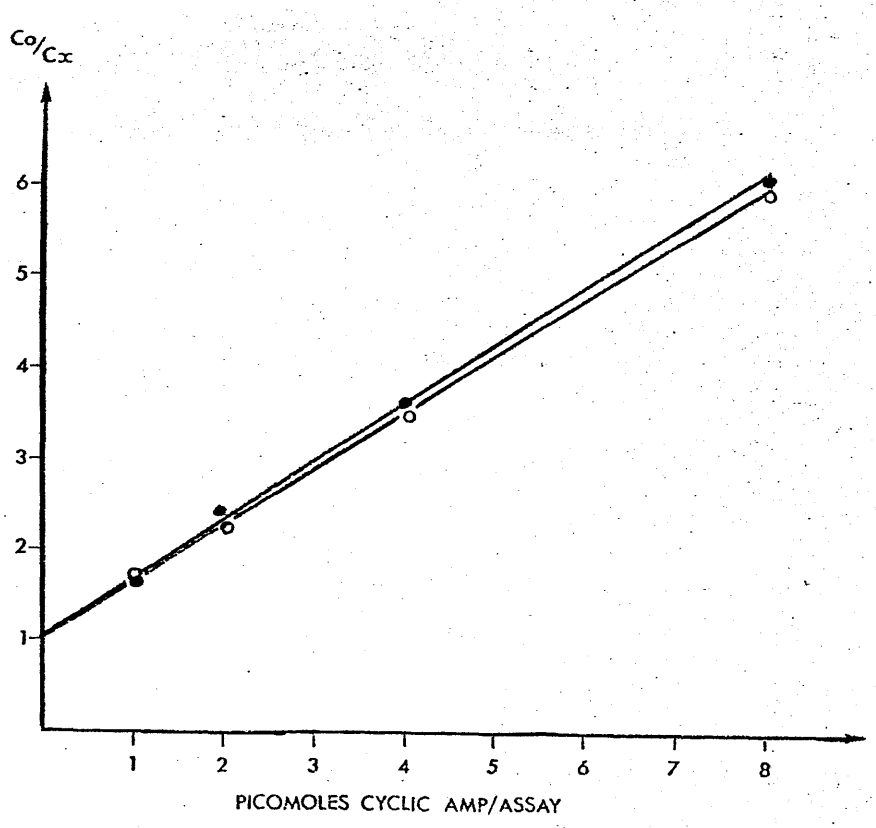
Having eliminated interference due to the extraction reagent employed, it was necessary to investigate interference originating from cell or incubation media constituents. Ideally, this involves using a cell extract where the components constitute intact cell extract except for the absence of cyclic AMP.

The addition of exogenous phosphodiesterase to samples indicated a decrease in precision resulting from its intro-

duction (Fig 12), an effect also found by Albano et al (1974). Therefore, cells were depleted of cyclic AMP using endogenous phosphodiesterase, by removing the medium from the dishes and incubating the cell sheet at 30°c for 48 hrs. TCA harvesting of these cells as described, including the ion-exchange chromatography step, gave rise to a reproducible standard curve with high precision and sensitivity (Fig 15). Maximum coefficient of variation obtained for standards diluted in the purified TCA extract was 7.2; maximum coefficient of variation for standards diluted in buffer was 6.5. Linear regressional analysis (Rees Midgeley et al, 1969) of amount predicted using the buffer curve versus amount measured using the cell extract curve, for the standard range 0.5 - 8 pmol cyclic AMP/assay gave a slope of 1.03 and an intercept of -0.01.

The final stage required in such authentication of the method was to validate the absence of cyclic AMP in the purified extract (ie zero). This was carried out by adding 30 ul (1% v/v) of assay charcoal suspension to the TCA supernatant followed by vigorous mixing and centrifugation. This step was considered a highly unlikely potential source of interference (of addition of exogenous phosphodiesterase) since the charcoal is used in the assay method itself. Under assay conditions, the charcoal is capable of adsorbing 99.5% of total free cyclic AMP. (Counts in the supernatant in the absence of binding protein, ie blank, constitute 0.5% of counts of total added ^{3}H-cyclic AMP. After treatment with charcoal, the extract yielded counts identical to background for a 50 µl "recovery" sample, ie the charcoal had removed all of the tracer. Charcoal addition did not affect the

FIGURE 15



Standard curve for standards diluted in H_2O (.) or in cell extracts depleted of cyclic AMP using endogenous phosphodiesterase and extracted using 5% TCA (o). Cell extracts were purified by ether extraction and Dowex ion-exchange chromatography. Control curve :r = 0.99. Cell extract curve :r = 1.00.

zero (To) value obtained for the cell extract blank and it was concluded that the sample had been depleted of cyclic AMP using endogenous phosphodiesterase. Hence TCA extraction including ion-exchange chromatography was used when harvesting cells for cyclic AMP assay.

Cells were harvested for cyclic AMP assay at 24 hr intervals from cell plating till the end of the four to five day growth period in ethanol containing medium.

Liquid Scintillation Counting

Cyclic AMP assay supernatants following charcoal addition and aliquots of cell extracts for recovery estimations were placed in plastic scintillation vials containing 10ml scintillant and counted using a Packard Tricarb model 2425 liquid scintillation spectrometer.

When counting aliquots of cell extracts for monitoring of recovery, blank values were obtained on parallel dishes using extraction agent minus ^{3}H-cyclic AMP tracer and counts obtained from these used as background estimations. Cyclic AMP assay blanks were obtained as described previously in methods (page 33).

A counting time of 10 mins was used for all samples. Counting error was never greater than 2%.

PART III

MEASUREMENT OF PROTEIN, RNA, DNA

Cells were harvested for protein, RNA and DNA assay at 24 hr intervals from cell plating till the end of each growh period in ethanol - containing medium. The experimental solution used to wash the cells for measurement of intracellular protein, RNA and DNA was Phosphate Buffered Saline (PBS). The constituents of PBS are 0.15M NaCl; 0.016M disodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) and 0.004M sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$). The pH of this solution is 7.4, minor adjustments being made, when required, by the addition of small quantities of HCl or NaOH.

Washing of Cells

Medium was aspirated from the cell monolayer, ice-cold PBS (10ml) was added to the cell sheet and the cells were resuspended in this using a glass rod with rubber stopper. For dishes containing less than 6×10^6 cells, two dishes were generally used and the cell suspension from the first dish transferred to the second. Cells were collected by centrifugation (500g; 2 mins) and the cell pellet resuspended in 10ml PBS. Centrifugation was rapidly repeated, then the cells were resuspended in 1.5-2ml PBS and counted using the Coulter Counter.

Estimation of Protein

Aliquots of washed cell suspension containing $2-4 \times 10^5$ cells were added to 100 μl IN NaOH. The solution was made up to 1ml then heated at 70°c for 30 mins to solubilise the

protein. Standards (0-200 µg protein/ml) prepared from a solution of crystalline Bovine Serum Albumin in 0.1N NaOH were similarly treated. The resulting hydrolysates were analysed directly for their concentration by the method of Lowry et al (1951). The colour intensities were read at a wavelength of 625nm using a Pye Unicam SP500 spectrophotometer.

Estimation of RNA and DNA

Aliquots of washed cell suspension, containing 2-4 x 10^6 cells were added to 200 µl 5M PCA and this cell extract made up to 2ml with distilled water. This was heated at 70°c for 30 mins to solubilise nucleic acid, then the protein precipitate was removed by centrifugation (3000g; 15 mins).

Supernatants were assayed for their content of RNA by a modification of the Orcinol Method (Ashwell, 1957). Aliquots (0.5ml) of supernatant were made up to 1.5ml with distilled water. To this was added 1.5ml 0.03%$FeCl_3$ in conc. HCl followed by 0.1ml 20% w/v orcinol in 95% ethanol. Tubes were mixed thoroughly and placed in a vigorously boiling water bath for 30 mins. RNA standards (0 to 100 µg/ml in 0.16M PCA), using RNA purified from yeast, were treated similarly. Extinctions were read at 625nm.

Supernatants (1ml aliquots) were assayed for their content of DNA by the method of Burton (1956). DNA standards (0 to 100 µg/ml in 0.5M PCA) using DNA purified from calf thymus, were used. Extinctions were read at 600nm.

MATERIALS

L-cells were obtained commercially from Gibco-Biocult Ltd, Renfrew, Scotland, as a trypsinised suspension. The growth medium (199), glutamine, foetal calf serum, Balanced Salt Solutions, trypsin and trypan blue concentrates used in the tissue culture procedures as well as the tissue culture dishes and bottles, were obtained from Gibco-Biocult.

Coulter Electronics Ltd, Herts, England supplied the "accuvette" containers and blow-out pipette for cell counting using the Coulter Counter.

Analar Grade reagents were used in the experimental solutions, obtained from British Drug Houses Chemicals Ltd, Poole, England. The ethanol used in experimental media (Absolute Alcohol, 99.9%) was also obtained from BDH.

The cyclic AMP assay kits were supplied by the Radiochemical Centre, Amersham, Bucks. Cyclic nucleotide phosphodiesterase and crystalline cyclic AMP were obtained from Sigma Chemical Company, London. The Dowex AG-50W x 8, 100-200 mesh cation exchange resin was obtained from Bio-Rad Laboratories, Richmond, California.

The scintillation fluid used in the counting of the ^{3}H isotope was NE260, supplied by Nuclear Enterprises Ltd Edinburgh.

Crystalline Bovine Serum Albumin, RNA and DNA were obtained from Sigma Chemical Company, London.

RESULTS

THE CELL CYCLE

L929 cells divide with a doubling time of 23.2 hours (Cleaver 1967). The orderly sequence of metabolic events occurring between the midpoint of mitosis and the midpoint of a successive mitosis constitute what is called the cell cycle. It is possible to divide the cycle arbitrarily into 4 phases. G_1. the period prior to DNA synthesis, S, the time of DNA replication and G_2, the period before the brief event of Mitosis, M. Fig 16 illustrates this diagramatically and shows the times of each period for L929 cells (Cleaver, 1967).

(a) Synchronous Growth in Culture

A culture is perfectly synchronised if all cells pass through a certain phase of their reproductive cycle at the same time.

(b) Asynchronous Growth in Culture

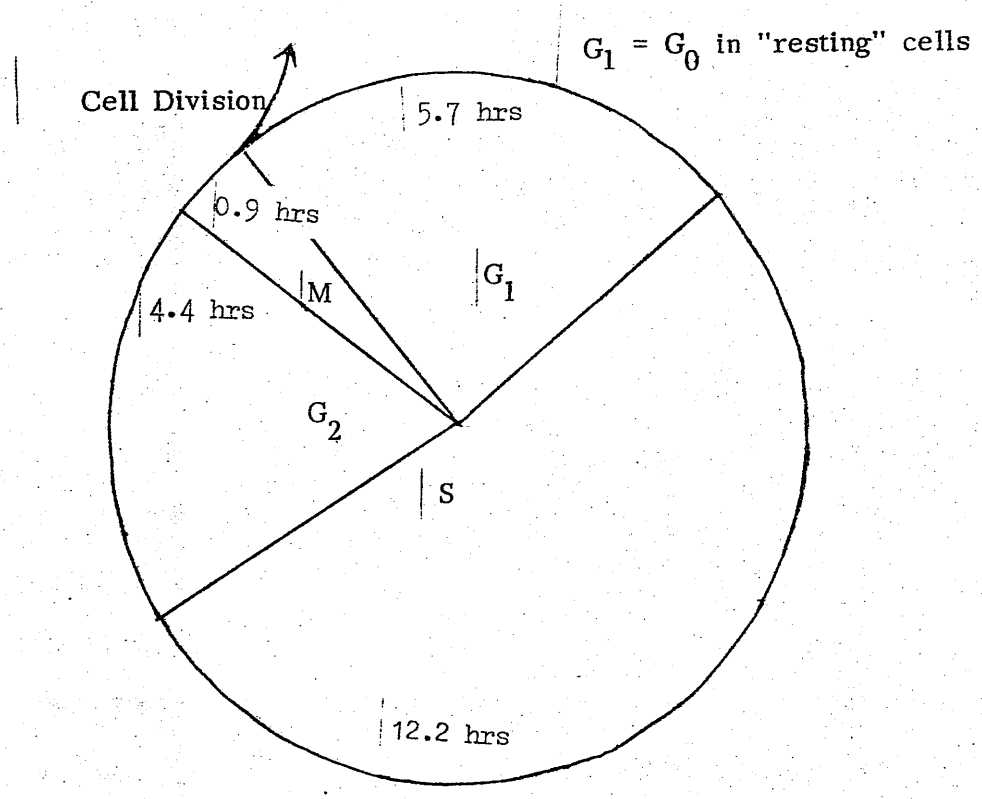
Asynchronous growth occurs if timing of cell division in a culture is perfectly randomised, with the cell number in the culture increasing exponentially in time.

A real culture may exhibit a behaviour intermediary to these extremes in that its growth pattern may be neither perfectly synchronous nor asynchronous.

Replating L929 Cells from Cultures at High Population Density

Cell cultures used in these experiments were seeded from parent cultures of cells which had been grown to a high population density and whose mitotic indices were low. Lindsay (1969) reported that the majority of cells in such densely-populated cultures are in the G_1 phase and have low DNA

FIGURE 16



The cell cycle for L929 cells.

polymerase activity. After subculture into fresh medium, entry into S phase was delayed a number of hours, confering a degree of synchrony upon the population. It was primarily assumed that such a system provided the basis for growth in my experiments, subject to verification by my findings.

As explained in the Methods section, cells were plated at densities of 2×10^6 and 3.5×10^6 cells per dish in two respective series of experiments; consequently these results are expressed in two parts (I and II). Growth of the cells is reported in terms of population per dish. Growth parameters in the form of DNA, RNA and protein are expressed in terms of their mass values for the cell population, as well as in terms of their intracellular concentrations. Cyclic AMP measurements are expressed in terms of its intracellular concentration. All results are related to time, from two days previous to, until five days after addition of ethanol-containing growth medium.

Part III reports immediate effects on intracellular cyclic AMP of addition of growth medium containing ethanol to mouse L-cells.

Calculation of Results

The Student's t-test was the statistical method employed to determine the significances of the results obtained.

PART I

ADDITION OF ETHANOL TO LAG PHASE CELLS

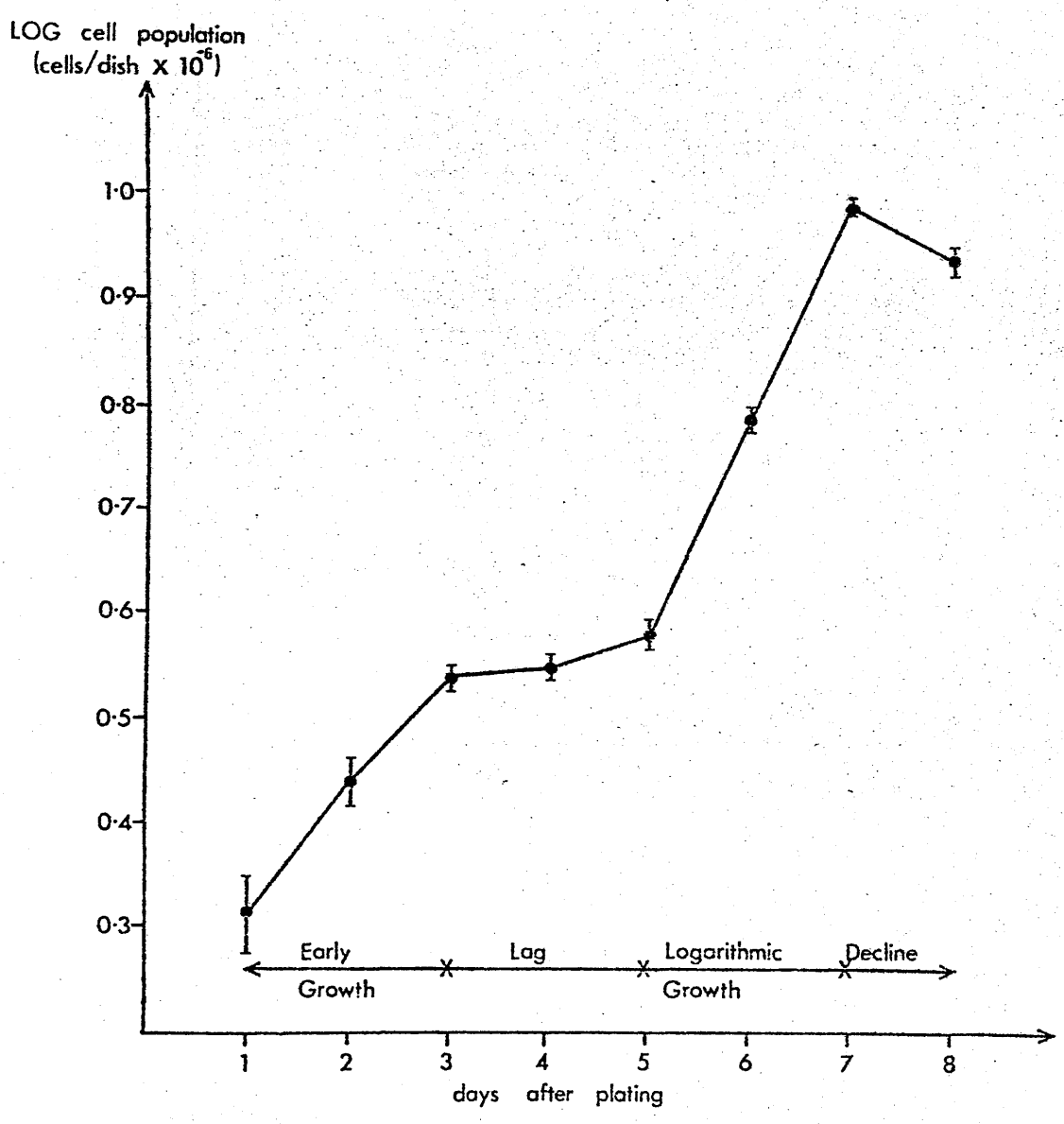
Cell Growth After Plating

In initial experiments, cells plated at a calculated cell density of 2×10^6 cells per dish grew as shown in Fig 17. The descriptions of the growth periods (early growth, lag, logarithmic growth, decline) which are illustrated in Fig 17 and subsequent figures, pertain to this thesis only.

Cell density on day 1 was 2.05 ± 0.17 (SEM) cells per dish $\times 10^{-6}$. This initial 24 hour period, in which little or no increase in cell number occurs, is a well documented phenomenon (Harris 1964; Paul 1970), and represents the time required for the cells to become adapted to their new environment (Harris 1964; Lindsay 1969). The graph of the logarithm of the cell numbers against days is linear from day 1 to day 3 (early growth period). Cell numbers increased by 34% to 2.75 ± 0.14 (SEM) cells per dish $\times 10^{-6}$, then by 26% to 3.46 ± 0.10 cells per dish $\times 10^{-6}$ over the 48 hour period from days 1 to 3. The cell population rose by only 10% over the 48 hour period from days 3 to 5. I have named this the lag period of growth. During this lag period in growth the number of cells lost from the dishes during medium changes was not significant. The fall in growth rate was a real phenomenon. The lag phase always occurred at a cell density of approximately 3.5 cells per dish $\times 10^{-6}$.

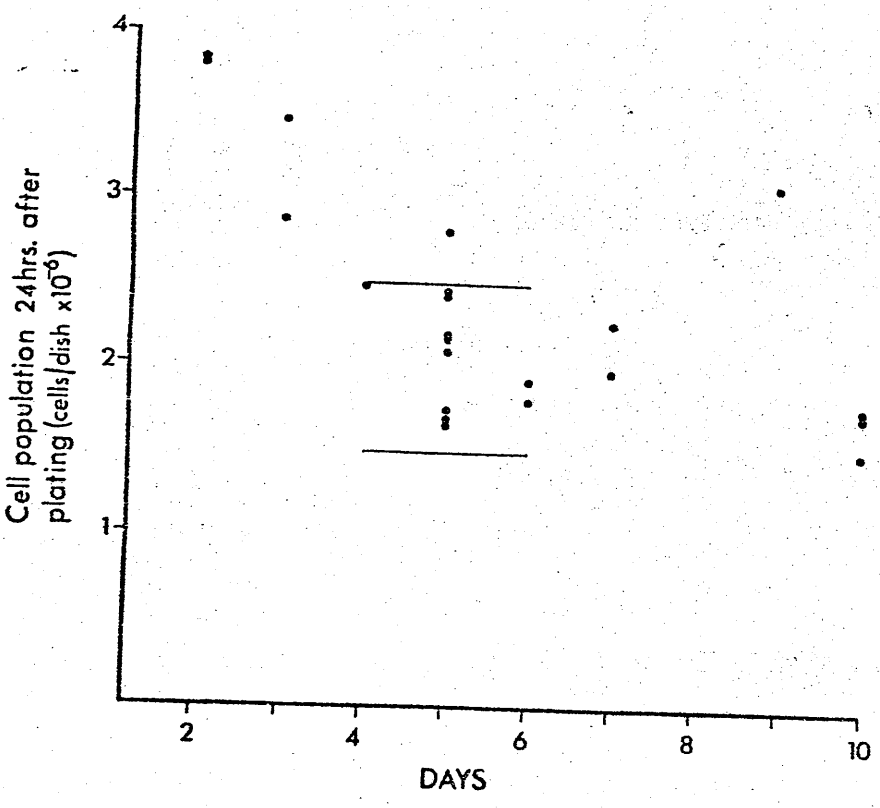
A two-day period of more rapid growth commenced on day 5 (logarithmic growth period). Number of days taken to reach logarithmic growth correlated with plating density ($r = 0.70$; $t = 4.22$; Fig 18). Only results from the area within the bars

FIGURE 17



Growth curve for L-cells plated at a cell density of 2 x 10^6 per dish. Points represent logarithm of the mean of 12 estimates; bars indicate ± SEM.

FIGURE 18



Cell populations per dish as measured 24 hours after plating, vs days taken to reach the logarithmic growth period. The bars indicate the experimental range which was used in the first series of experiments; ie populations which grew logarithmically between 4 and 6 days after plating and whose cell densities 24 hours after plating were between 1.6 and 2.4 cells per dish x 10^{-6}.

in Fig 18 were considered, ie cells which entered logarithmic growth between four and six days after plating. Cell numbers increased by 62% and 59% respectively over the two 24 hour periods between days 5 and 7. By day 8, cell numbers had become static at a cell density of 9.80 ± 0.25 cells per dish x 10^{-6}. L929 cells plated at the density of 2.0×10^{6} cells per dish could be maintained for ten days or more with little or no detachment of cells from the plates. Thus between days 7 and 8, a real decline in growth rate had occurred.

Investigators have generally reported the growth curves of mammalian cell lines as showing a period of little or no cell division during the first 24 hours after plating, followed by a logarithmic phase and finally a decline (Paul 1970). The lag period which occurred between days 2 and 4 in my experiments corresponded to a cell density of 4.8×10^{4} cells per cm^{2}. Such a density corresponds to formation of a complete monolayer and cessation of growth in non-transformed cell lines, eg 3T3 (Bannai and Sheppard 1974) and astrocyte-like cells from normal adult brain (Pontén et al 1969).

Metabolic Fate of Ethanol After Addition to the Growth System

Blank dishes, without cells, containing growth medium plus ethanol, were included in all experiments. The decrease in concentration of ethanol as a result of evaporation (20-25% per twenty-four hours) was identical in both blank and experimental dishes. No attempt was made to find evidence of metabolic degradation of ethanol by L929 cells.

Effect of Ethanol on Cell Population

When ethanol was added at a cell density of 3.5 ± 0.1 (SEM) cells per dish x 10^{-6}, its addition had no effect on cell population during the 48 hour lag period immediately following (Fig 19). During the first day of logarithmic growth, cell numbers in dishes containing 21mM and 42mM ethanol increased by 38% and 17% respectively whereas control cell populations increased by 62% ($t = 2.54$; $p < 0.01$ and $t = 5.31$; $p < 0.0005$ respectively for 21mM and 42mM ethanol containing media). This effect of ethanol could have resulted from:

(i) a decreased growth rate, or

(ii) an increased lag phase

during the first 24 hours of the logarithmic growth phase.

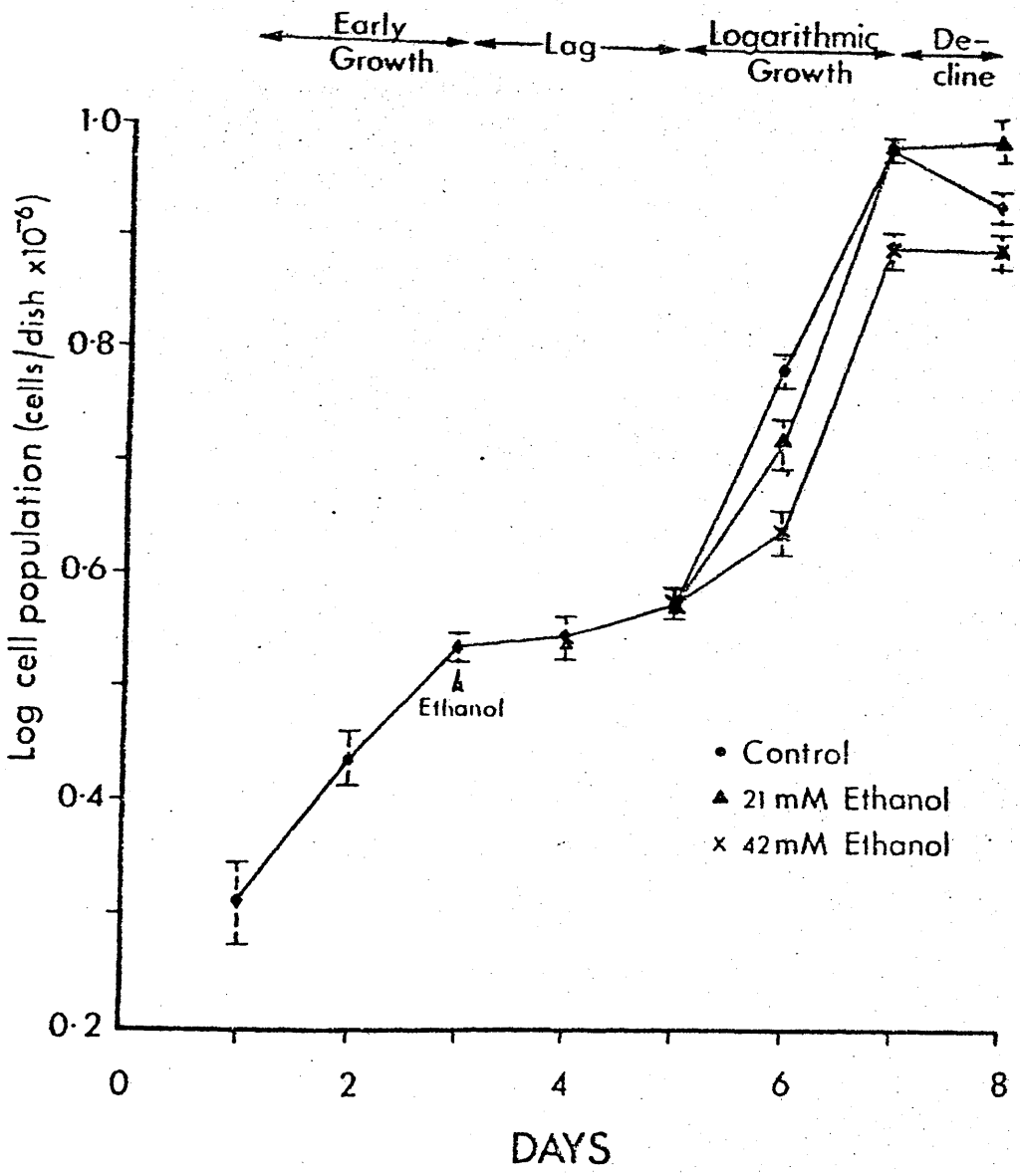
During the latter 24 hours of logarithmic growth, cell population increased by 87% and 80% respectively in dishes containing 21mM and 42mM ethanol, whereas cell population increased by 59% in control dishes. Thus during the latter half of the logarithmic growth phase ethanol either:

(i) increased the growth rate of the cells or

(ii) caused them to divide more synchronously than control cells.

Ethanol did not affect the timing of growth decline. The decline in growth rate occurred at a lower cell density in 42mM ethanol treated cells (7.94 ± 0.31 cells per dish x 10^{-6}; $t = 4.71$; $p < 0.0005$). In 21mM ethanol the growth decline occurred at the same cell density as in control dishes.

FIGURE 19



The effect of ethanol on cell population. Ethanol-containing growth medium was added on day 3, at a cell population of 3.5 ± 0.1 (SEM) cells per dish x 10^{-6}. The medium was changed daily thereafter. Results are mean of 12 experiments; bars indicate SEM.

THE CYCLIC AMP ASSAY

1 VALIDATION OF THE METHOD

Considerable discussion within the Methods section has already been devoted to validation of 5% TCA as extraction agent, followed by ether extraction and ion-exchange chromatography. Other parameters of assay validity: precision, accuracy and sensitivity, were quantitated for the method.

Within-Assay and Between-Assay Precision

Cell extracts (samples) were assayed by the technique described in the Methods section. Within-assay precision was determined as the coefficient of variation obtained from replicate determinations of the same sample. Between-assay precision was calculated on replicate samples assayed in duplicate on separate occasions over a period of seven weeks. The results were compared with those of standard cyclic AMP in buffer. Tables VIII A and VIII B illustrate the findings.

Precision of cyclic AMP measurements for samples was not significantly different to that of standards within or between assays ($p > 0.3$). The method can be used with a within-assay coefficient of variation of 17.5% or less over the range 0.4-4.0 pmol/50 µl sample. This is greater than the maximum coefficient of variation of 11% reported by the manufacturers (Tovey et al 1974) and was found to originate from at least two effects:

(i) The efficiency of the charcoal separation step tended to decrease progressively (by 0.3-0.4% during the

TABLE VIII

(A) Within-Assay Precision

pmol cyclic AMP per assay ±SD	Within-assay Precision (coefficient of variation)	No of determinations
Standards		
0.60±0.11	18.3	7
1.05±0.15	14.3	10
1.95±0.30	15.4	8
4.25±0.50	11.8	10
7.48±0.92	12.3	9
Samples		
0.40±0.07	17.5	10
1.11±0.16	14.4	8
1.98±0.32	16.0	9
3.89±0.44	11.3	9

(B) Between-Assay Precision

pmol cyclic AMP per assay ±SD	Between-assay Precision (coefficient of variation)	No of assays
0.49±0.09	18.4	10
1.03±0.17	16.5	10
2.03±0.22	10.8	11
3.97±0.35	8.8	10
7.72±1.10	14.3	10
Samples		
0.40±0.07	17.5	11
1.23±0.19	15.5	10
2.03±0.41	20.2	11
4.12±0.42	10.2	9

two-hour period of its use in an average assay containing 100 tubes). Decreased efficiency of adsorption probably originated from denaturation of the protein solution in which the charcoal is suspended due to heat generation from the magnetic stirring apparatus. Precision could be improved by including a standard curve at the beginning, middle and end of an assay.

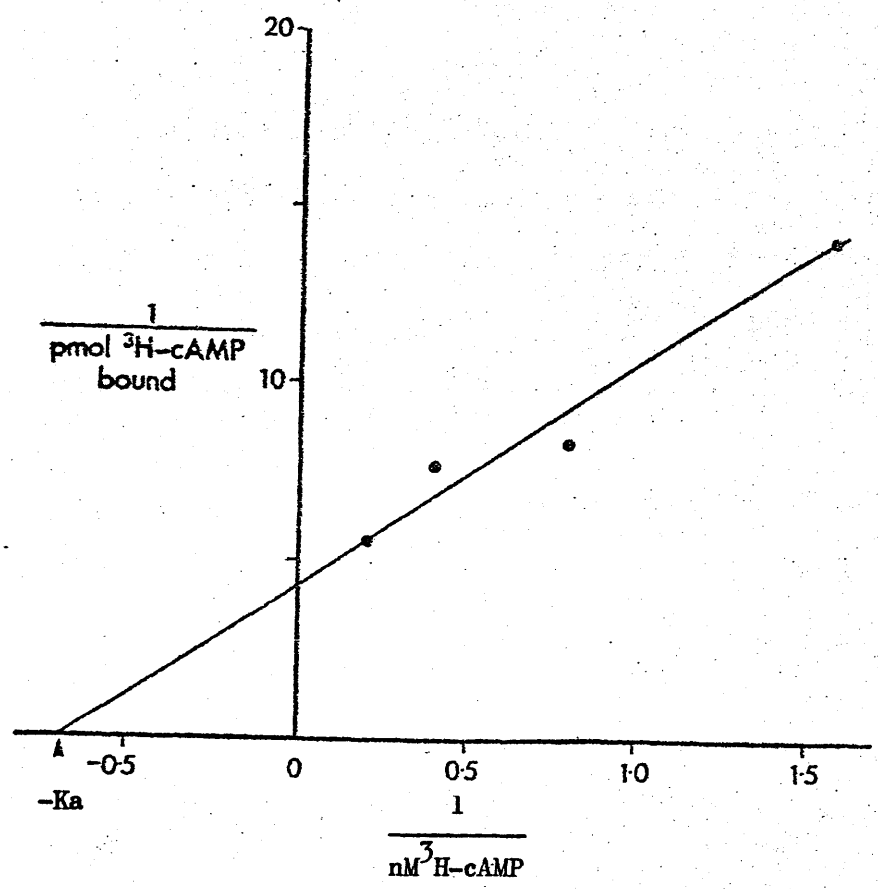
(ii) The slope of my standard curve was less than that reported by the manufacturers. Standard curves in my laboratory had a mid-range, ie the dose required to reduce the response to half its initial value ($^{Co}/Cx = 2$), of 1.5-1.6 pmol cyclic AMP per 50 µl sample (Figs 12 to 15). Tovey et al (1974) reported a mid-range of 1.2-1.3 pmol cyclic AMP per 50 µl sample. I calculated the association constant of the binding protein for cyclic AMP by a double recriprocal plot (Fig 20). I found the association constant to vary between batches of kits (from 0.62 to 0.84 x 10^{-9} M^{-1} on samples measured). This is lower than the value of 1.0 x 10^{-9} M^{-1} reported by Tovey et al (1974) and could easily account for the diminished slope of the standard curve.

Within batches, precision could not be further improved by increasing assay incubation time, staggering addition of ^{3}H-cyclic AMP and protein, or increasing counting times in the liquid scintillation counter and was not investigated further.

Accuracy of the Method

This was determined by adding a range of cyclic AMP standards (0.25 to 8 pmol per assay) to 3 ml samples of the cell extract blank of TCA. The resultant mixture was assayed by the technique described in the Methods section.

FIGURE 20



Typical double reciprocal plot for determination of the association constant for binding of cyclic AMP to the binding protein. The binding protein concentration used was 7.5 µg/ 200 µl incubation volume; which is x1.5 the concentration used in a normal assay. This plot calculates the association constant (Ka) to be 0.65 x $10^{-9}M^{-1}$. Each point is average of 4 determinations.

Correction for losses during extraction were calculated from recovery of ^{3}H-cyclic AMP tracer. The results are presented in Table IX.

The recovery of added cyclic AMP between 0.5 and 8 pmol per assay was at least 97.3%. This involves an error of 2.7% at most for any sample, which is well within the precision limits of the assay. However, accuracy was considerably decreased at the lower limit of detection. For samples containing 0.25 pmol cyclic AMP per assay, the error between the calculated and "true" result was 44%, which is outwith the precision limit of the assay.

Sensitivity of the Method

As Table IX shows, samples containing 0.5 pmol cyclic AMP per assay or more could be recovered with adequate accuracy. Within-assay coefficient of variation for this standard was 16.5. This was within the within-assay precision limits of standards (Table VIII A) Thus the method was considered sensitive to 0.5 pmol cyclic AMP per assay.

TABLE IX

Accuracy of the Method

pmol cyclic AMP		Accuracy (Recovery %)	No of Determinations
Added	Measured (±SD)		
0.25	0.36±0.18	144.0	8
0.50	0.49±0.20	98.2	6
1.00	1.00±0.08	100.0	6
2.00	1.96±0.24	98.0	6
4.00	3.89±0.27	97.3	7
8.00	7.81±1.34	97.6	6

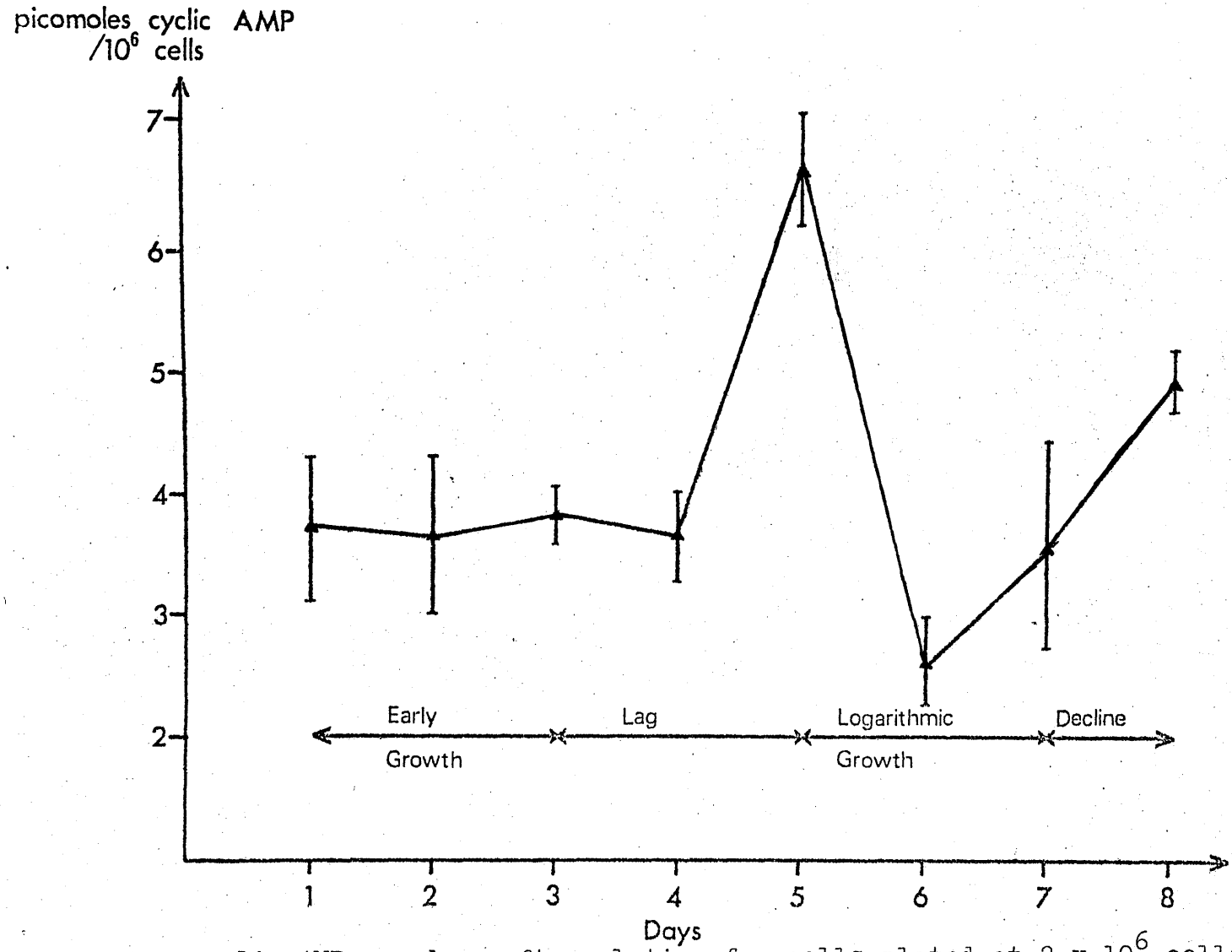
2 INTRACELLULAR CYCLIC AMP THROUGHOUT GROWTH

Fig 21 illustrates average intracellular cyclic AMP throughout the experimental period. Little change in cyclic AMP content occurred during early growth and the initial 24 hours of the lag period. Mean intracellular cyclic AMP from days 1 to 4 was 3.71 ± 0.17 (SEM) pmol/10^6 cells. During the latter half of the lag period, cyclic AMP rose significantly ($t = 3.98$; $p < 0.0025$) from 3.64 ± 0.39 to 6.68 ± 0.41 pmol/10^6 cells (an increase of 84%). On the other hand, between days 5 and 6, its cellular content fell by 70% ($t = 7.27$; $p < 0.0005$) reaching a minimum 24 hours after logarithmic growth commenced. On day 6, cellular cyclic AMP was lower than the mean basal level from days 1 to 4 ($t = 3.09$; $p < 0.005$).

In the second day of logarithmic growth, cyclic AMP increased by 37% to 3.53 ± 0.81 pmol/10^6 cells. Scatter of results was high on day 7 (standard deviation was 46% of the mean; $n = 10$) suggesting that cellular cyclic AMP fluctuates during logarithmic growth. Cyclic AMP increased further during the growth decline phase, to 4.91 ± 0.21 pmol/10^6 cells.

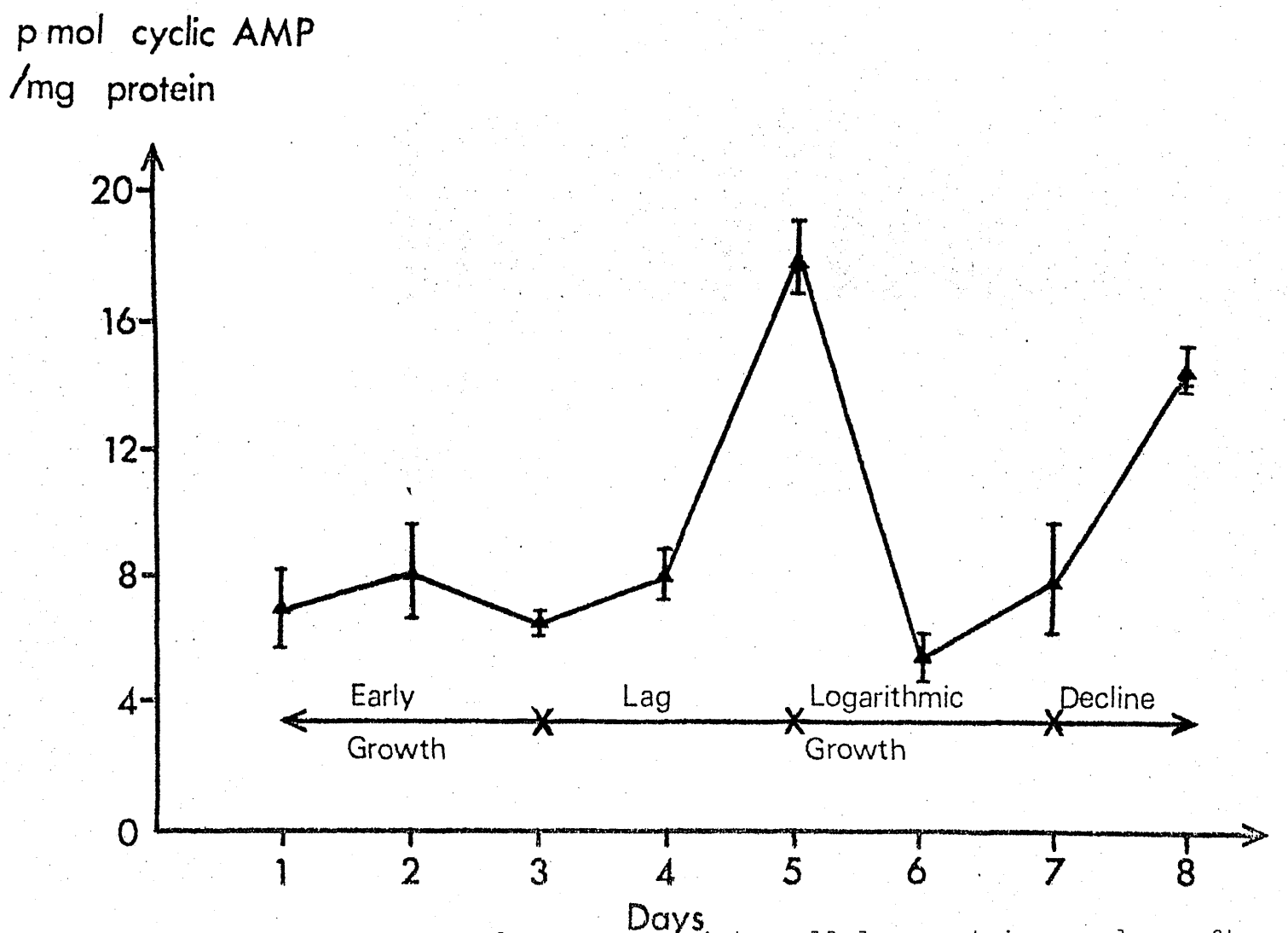
To preclude the possibility that changes in cell volume during the growth period influenced intracellular cyclic AMP concentrations, cyclic AMP levels were expressed as per milligram protein. A large proportion (80%) of the mammalian cell comprises of protein (MacKinnon 1969) which may be used as an indication of cell volume. Cyclic AMP per mg protein is illustrated in Fig 22. The same qualitative changes may be seen: a mean basal level of 7.49 ± 0.46 pmol cyclic AMP per mg protein during early growth and early lag, rising significantly

FIGURE 21



Intracellular cyclic AMP, vs days after plating for cells plated at 2 x 10^6 cells per dish. Each point is the mean of at least 8 determinations; bars indicate $\pm$ SEM.

FIGURE 22



Intracellular cyclic AMP, expressed as per mg intracellular protein, vs days after platig, for cells plated at 2 x 10^6 cells per dish. Each point is the mean of 8 determinations; bars indicate ± SEM.

($t = 6.16$; $p < 0.0005$) to 18.05 ± 1.11 pmol per mg protein during the latter 24 hours of the lag period; a fall from 18.05 ± 1.11 to 5.45 ± 0.81 pmol per mg protein ($t=9.18$; $p < 0.0005$) during the first 24 hours of logarithmic growth. Furthermore the rise in cyclic AMP per mg protein, from 7.92 ± 1.82 at late logarithmic growth (day 7) to 14.60 ± 0.62 on day 8 was statistically significant ($p < 0.05$)

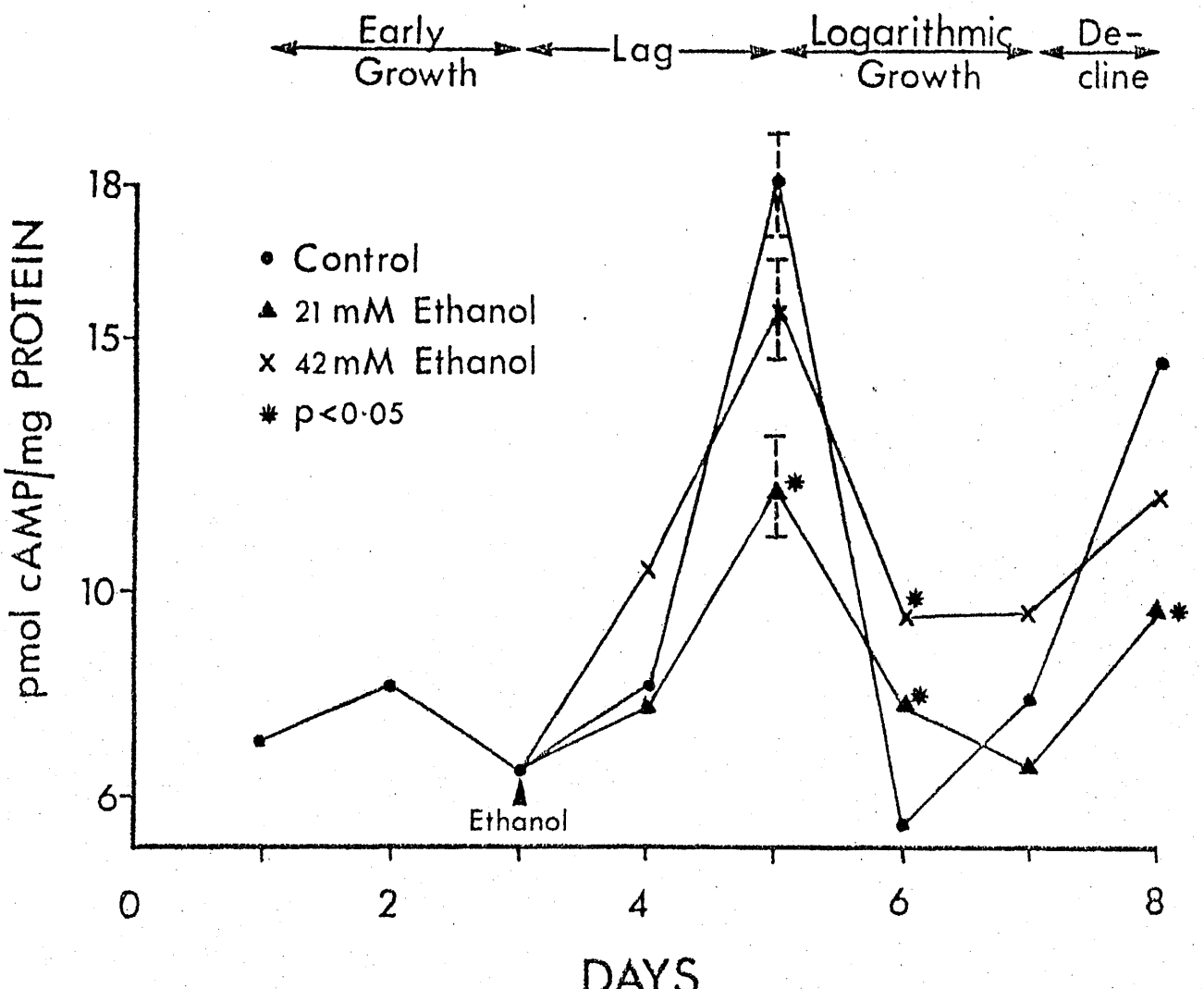
Effect of Ethanol on Intracellular Cyclic AMP Throughout Growth

In cells incubated with ethanol from day 3, cyclic AMP also rose at the end of the lag phase (Fig 23). On day 5, ethanol treated cells tended to have lower intracellular cyclic AMP per mg protein; in 21mM ethanol treated cells the content of 12.05 ± 1.05 pmol cyclic AMP per mg protein was significantly lower than in control cells ($t = 2.73$; $p < 0.005$).

Midway through the logarithmic growth period in control cells (day 6) cyclic AMP levels in experimental cells fell to 7.77 ± 0.62 pmol per mg protein for 21mM ethanol treated cells and 9.54 ± 1.00 pmol per mg protein for 42mM ethanol treated cells. These levels were thus significantly higher than the mean of 5.45 ± 0.81 pmol cyclic AMP per mg protein measured in control cells ($t = 2.36$; $p < 0.025$ and $t = 1.69$; $p < 0.05$ for cells grown in 21mM and 42mM ethanol respectively). At growth decline on day 8, ethanol treated cells tended to have lower intracellular cyclic AMP.

Thus the alteration in growth pattern which occurred when ethanol was added to the growth medium of L929 cells was parallelled by an increased intracellular cyclic AMP in these cells over control. Interpretation of the results following this period (days 6 to 8) is difficult due to the fact that the growth states of control and ethanol treated cells may have differed.

FIGURE 23



The effect of ethanol on intracellular cyclic AMP for cells plated at 2 x 10^6 cells per dish. Ethanol containing growth medium was added on day 3 and changed daily thereafter. On day 5, when logarithmic growth was occurring in control cells the decreased growth rate or extended lag in ethanol treated cells (see Fig 19) was parallelled by increased intracellular cyclic AMP. Results are mean of 10 experiments; typical SEM bars shown.

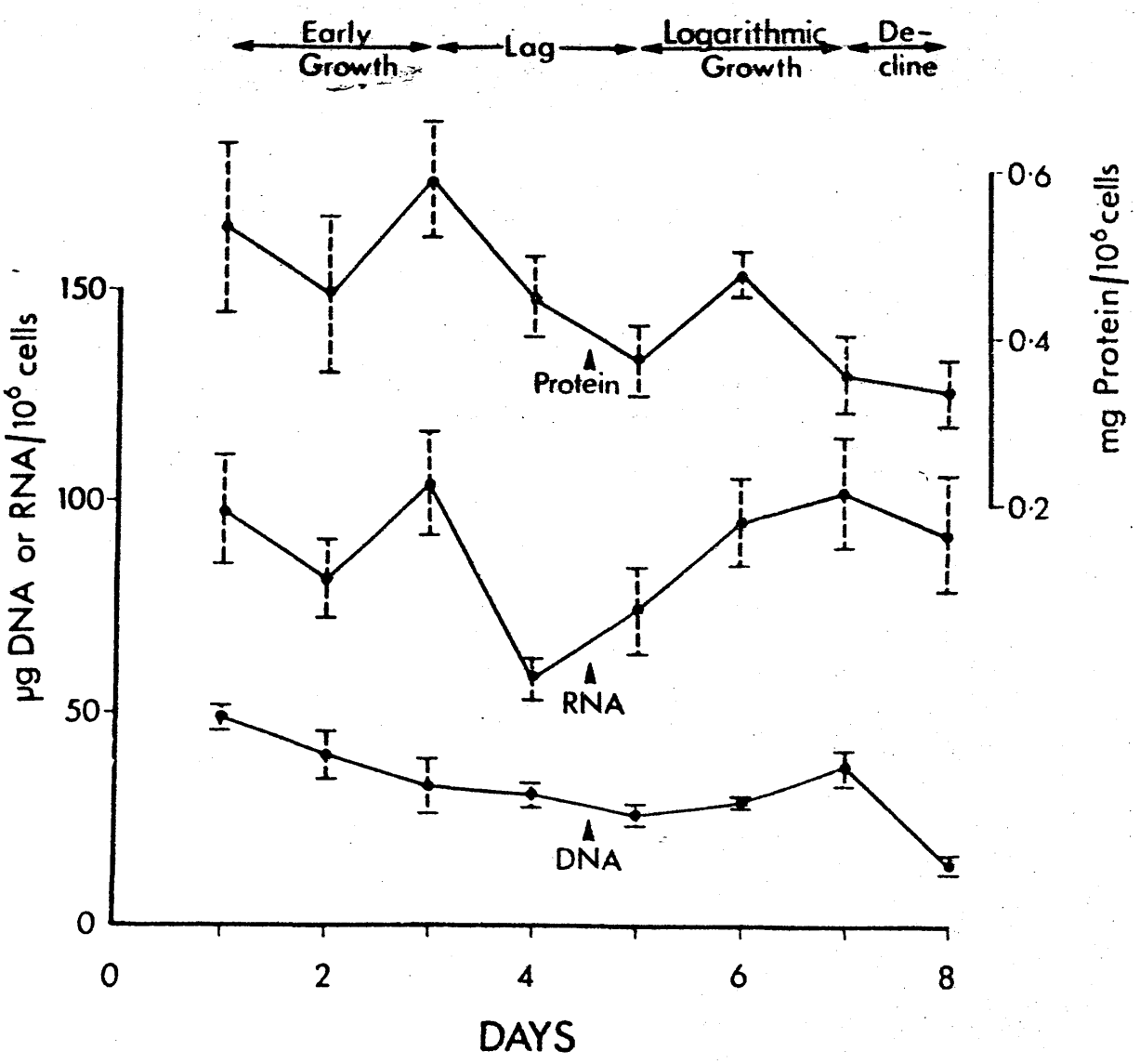
CHANGES IN DNA, RNA AND PROTEIN DURING GROWTH

Concentrations of intracellular DNA, RNA and protein 24 hours after plating were 48.7±2.9 (SEM) μg per 10^6 cells; 98.0±19.8 μg per 10^6 cells and 0.525±0.105 mg per 10^6 cells respectively (Fig 24). In the cases of DNA and protein, these concentrations tended to be higher than those of logarithmically growing and decline phase cells. The concentration of RNA tended to be the same or of higher magnitude than that of logarithmically growing and decline phase cells. These results imply that the cells had synthesised DNA, RNA and protein during the time interval between trypsinisation and 24 hours after plating out. This phenomenon has been reported by others (Swaffield and Foley, 1960; Ward and Plagemann 1973) and is comparable to bacterial growth (Salzmann 1959). Raising external $[K^+]$ has been shown to block BHK cells in the G_1 phase of the cell cycle. Concurrent with this was increased intracellular DNA, RNA and protein. (Orr et al 1972).

DNA

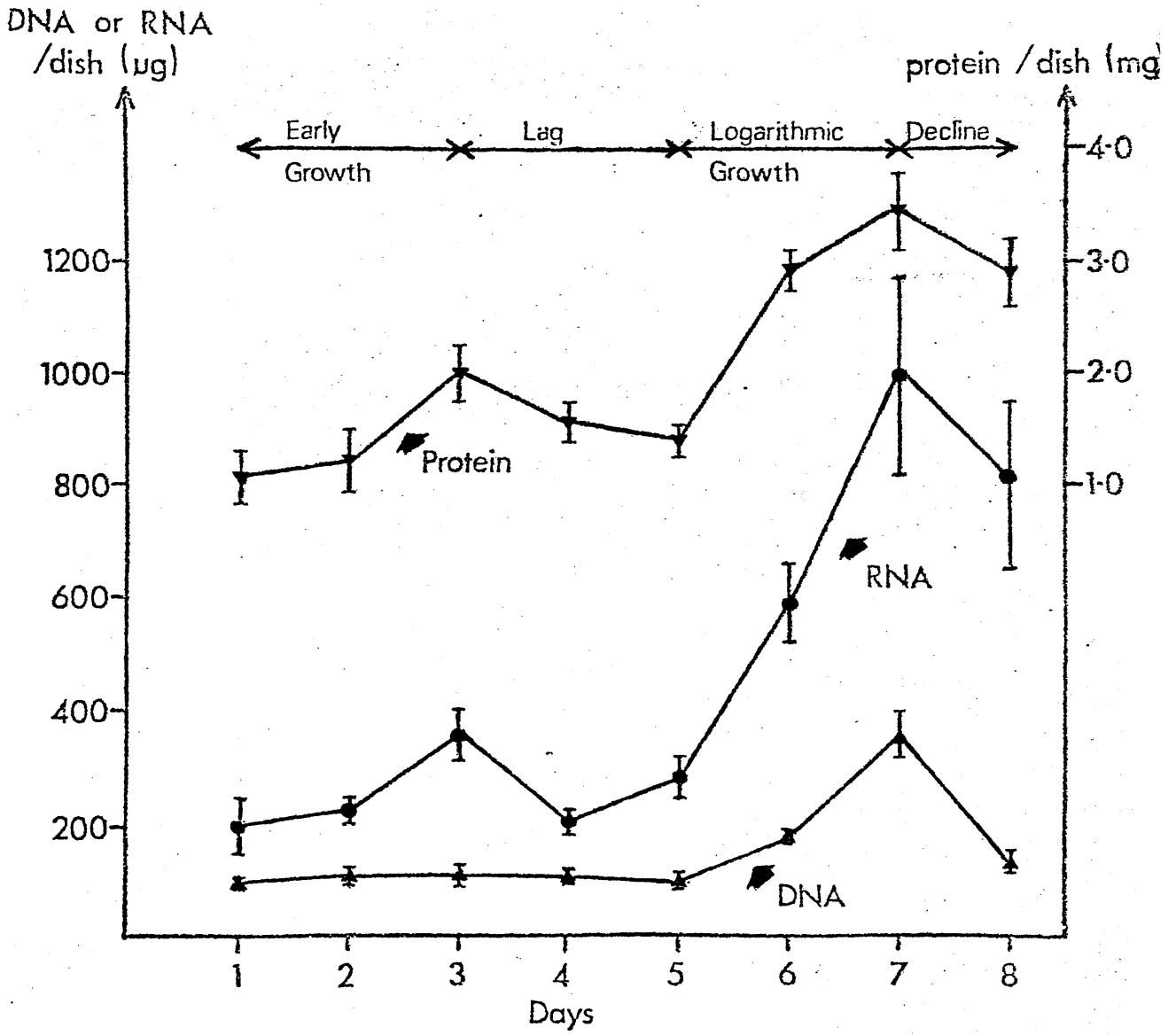
As indicated in Fig 25 , DNA per dish increased between days 1 and 2 (by 11%). In the presence of cell division between days 1 and 2, this resulted in a decrease of 18% in DNA per cell (Fig 24). From days 2 to 3, total DNA content per culture did not change and cell DNA content continued to fall. During the lag period in growth, there was a 13% fall in DNA per dish ($p > 0.2$). These results

FIGURE 24



Intracellular DNA, RNA and protein from 1 to 8 days after plating, for L-cells plated at 2 x 10^6 cells per dish. Measurements were obtained on washed cells, as described in Methods. Each point represents the mean of 10 measurements; bars indicate ± SEM.

FIGURE 25



Total culture DNA, RNA and protein from 1 to 8 days after plating, for L-cells plated at 2 x 10^6 cells per dish. Estimates were calculated on measurements of intracellular DNA, RNA and protein of washed cells, obtained as described in Methods, multiplied by the cell population per dish. Each point represents the mean of 10 estimates; bars indicate ± SEM.

are consistent with the findings of Lindsay (1969), who reported that the trypsinisation procedures or cooling of L-cells during initiation of cultures prevented them temporarily from entering S phase (although the delay reported by Lindsay was only a matter of hours). The rises of 10.5% and 2.7% respectively in DNA per dish over the two 24 hour periods of the early growth phase were parallelled by rises in cell population of 34% and 26% respectively. This implies that some cells were in G_2 on day 1. These cells progressed through mitosis to G_1 during days 1 to 3, but did not enter S phase until day 5.

Logarithmic growth was parallelled by an increasing DNA content per culture. DNA decreased per dish as well as per cell during the growth decline period, in agreement with Lindsay (1969) who reported a decrease in DNA polymerase activity from late logarithmic growth onwards in L-cells.

The DNA changes, per cell and per dish, qualitatively parallel those of Newton and Wildy (1959). These investigators reported that exposure of cultures of HeLa cells to a temperature of 4% for 60 mins was followed, some time after replacement of the cells at 37% by a synchronous increase in cell number.

RNA

Between days 1 and 2, a slight (12%) increase in RNA per dish occurred in the presence of cell division, resulting in a slight (17%) fall in RNA per cell (Fig 24). Between days 2 and 3, a rise occurred of 61% ($t = 2.44$; $p < 0.025$) per dish in RNA (Fig 25). Such a rise in RNA per culture, together with a rise in cell numbers but no increase in DNA per culture is consistent with cells progressing from

G_2 to G_1, since protein and RNA synthesis continues during G_2, M and G_1 in the absence of DNA syntheis (Baserga 1965). From days 3 to 4 (early lag) a 43% fall in RNA per dish in the absence of an increase in cell population resulted in a significant ($t = 4.54$; $p < 0.0005$) fall in cellular RNA, from 104.4 $\mu g/10^6$ cells to 58.3$\mu g/10^6$ cells. During the latter half of the lag phase, RNA per dish increased by 37% ($t = 1.86$; $p < 0.05$) in the absence of an increase in DNA or protein. With the 7% increase in cell numbers between days 4 and 5, this resulted in a 28% increase in intracellular RNA, from 58.3 ± 5.4 $\mu g/10^6$ cells to 74.5 ± 10.2 $\mu g/10^6$ cells ($t = 1.47$; $p < 0.05$). Thus RNA synthesis preceeded DNA synthesis before commencement of logarithmic growth. This occurrence has been found by many investigators; (Lieberman et al 1963b; see Baserga 1965) and is consistent with an enzyme induction mechanism being involved in initiation of the DNA synthesis, which increases at logarithmic growth (Weissman et al 1960).

During logarithmic growth, RNA continued to increase, per culture as well as per cell. RNA fell per cell and per dish during the growth decline phase, in agreement with Ward and Plagemann (1973) who found a decreasing RNA polymerase activity from late logarithmic growth onwards in Novikoff rat hepatoma cultures.

Protein

Between days 1 and 2 protein per dish increased (by 13%; Fig 25). Since cell division occurred between days 1 and 2, this resulted in a decrease of 15% in protein per cell (Fig 24). Between days 2 and 3, a rise occurred of 66% in

protein per culture. This event is consistent with the evidence (above) that a proportion at least of the cells were progressing from G_2 to G_1 during the early growth period. During the lag period in growth, there was a 31% fall in protein per dish ($t = 2.08$; $p < 0.05$). Protein also fell per cell, from 0.585 ± 0.027 mg/10^6 cells on day 3, to 0.370 ± 0.042 mg/10^6 cells on day 5: a fall of 37% ($t = 2.57$; $p < 0.01$).

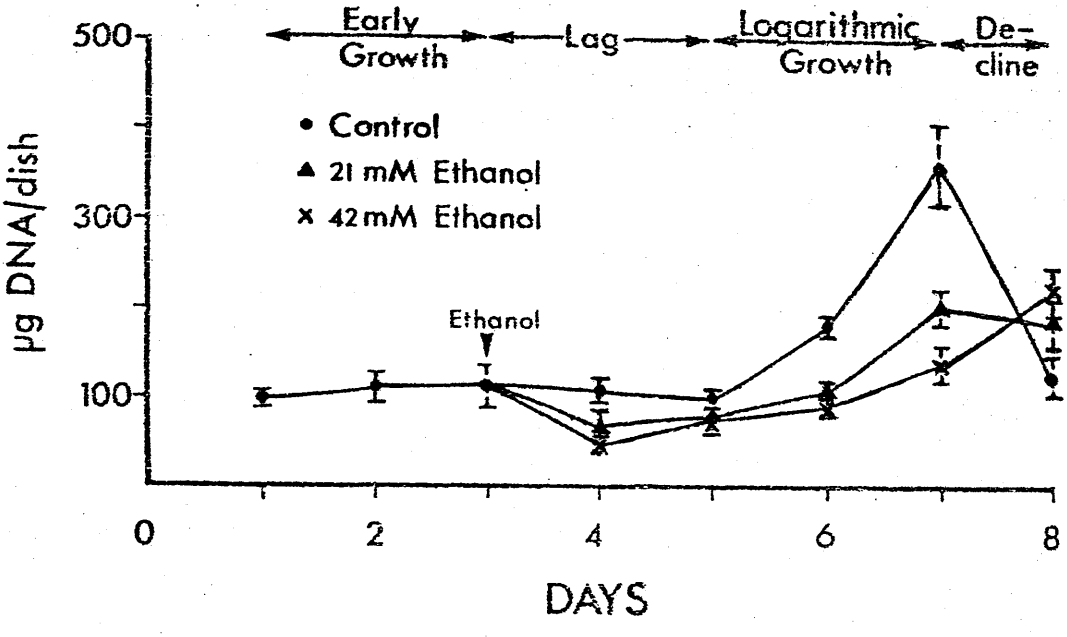
After an initial rise in protein per cell and per plate during early logarithmic growth, the rate of increase in protein per dish fell during the mid-late logarithmic growth phase (decreased slope of line; Fig 25) and protein per cell fell. This result might suggest that a drop in intracellular protein may be a primary event related to timing of growth decline.

The results above are consistent with the findings of Lindsay (1969) in that entry into S phase is delayed in cells replated from high population density cultures. Such an explanation is likely despite variations in the time length of this delay (Lindsay 1969, personal communication) and such an occurrence is especially the case when cooling procedures are included (Newton and Wildy 1959). As described in the Methods section, cells obtained commercially were shipped in refrigerated containers.

Effects of Ethanol on DNA

Addition of ethanol resulted in a decreased DNA content per plate with 24 hours of its addition ($t = 2.19$; $p < 0.05$ and $t = 3.75$; $p < 0.0025$ respectively for 21mM and 42mM ethanol treated cells; Fig 26). Since this effect occurred in the absence of any change in cell population, DNA content per cell was apparently lower in ethanol treated cells ($t = 2.21$; $p < 0.025$; Table X).

FIGURE 26



The effect of ethanol on culture DNA for cells plated at 2 x 10^6 cells per dish. Ethanol-containing growth medium was added on day 3 and changed daily thereafter. Results are mean of 10 experiments; typical SEM bars shown.

TABLE X

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days after Plating	DNA ($\mu g/10^6$ cells)		
1	48.7 ± 2.9	-	-
2	40.1 ± 5.6	-	-
3	32.8 ± 6.5	-	-
4	30.7 ± 2.5	19.7 ± 4.1	13.6 ± 1.7
5	26.0 ± 2.4	20.5 ± 4.6	19.6 ± 2.2
6	28.8 ± 1.4	20.3 ± 1.9	20.4 ± 3.2
7	37.4 ± 4.2	20.2 ± 2.3	17.4 ± 2.1
8	14.6 ± 2.0	18.5 ± 3.5	27.0 ± 4.3

Intracellular DNA during the growth cycle of cells plated at a density of 2×10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Growth medium was changed daily from day 3 onwards in all dishes, as described in Methods. Results are average of 10 experiments.

During the latter 24 hours of the lag phase, DNA per dish rose slightly in ethanol-treated cells (13% and 56% in 21mM and 42mM ethanol treated cells respectively). This occurred in the presence of a 9% fall in DNA content per dish in control cells. On day 4 (mid lag) intracellular DNA content of 21mM and 42mM ethanol treated cells were 64% ($t = 2.21$; $p < 0.025$) and 44.3% ($t = 3.70$; $p < 0.0025$) respectively of control cells. By day 5 (late lag) cell numbers had not changed significantly, but DNA content of ethanol treated cells was more than 75% of control cells.

As logarithmic growth commenced in control cells (days 5 to 6) there was a 79% rise in DNA per dish but rises of only 36% and 21% respectively in 21mM and 42mM ethanol treated cells. As described above, this was associated with delay in the increase of cell division and raised intracellular cyclic AMP. Throughout the lag and logarithmic growth phases, ethanol treated cells had lower DNA, expressed either per dish or per cell, than control cells.

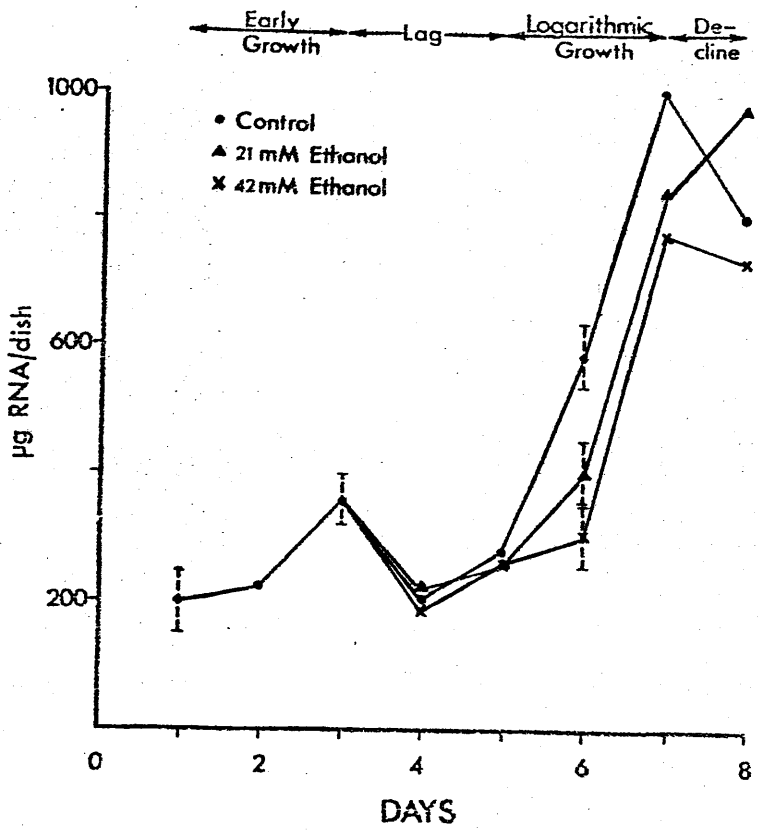
Interpretation of the results after day 6 is difficult as growth states of control and ethanol treated cells markedly different.

Effects of Ethanol on RNA

On days 4 and 5, RNA content in ethanol treated cells was not significantly different from control, either expressed per dish ($p > 0.2$; Fig 27) or per cell ($p > 0.2$; Table XI). Although ethanol treated cells tended to have lower RNA per dish at the end of the lag period (day 5) ethanol apparently had not prevented the rise in RNA which preceeded the rise in DNA before logarithmic growth.

After 24 hours of logarithmic growth in control cells

FIGURE 27



The effect of ethanol on culture RNA for cells plated at 2 x 10^6 cells per dish. Ethanol-containing growth medium was added on day 3 and changed daily thereafter. Results are mean of 10 experiments; typical SEM bars shown.

TABLE XI

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days after Plating	RNA ($\mu g/10^6$ cells $\pm$ SE)		
1	98.0±15.5	-	-
2	81.8±9.1	-	-
3	104.4±12.1	-	-
4	58.3±5.4	61.7±5.0	55.0±7.1
5	74.5±10.2	70.1±11.3	69.0±13.0
6	95.6±11.8	76.3±8.8	69.1±11.2
7	103.0±17.1	78.3±10.0	98.0±16.9
8	93.0±16.3	99.2±16.5	90.7±17.5

Intracellular RNA during the growth cycle of cells plated at a density of 2 x 10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Results are average of 10 experiments.

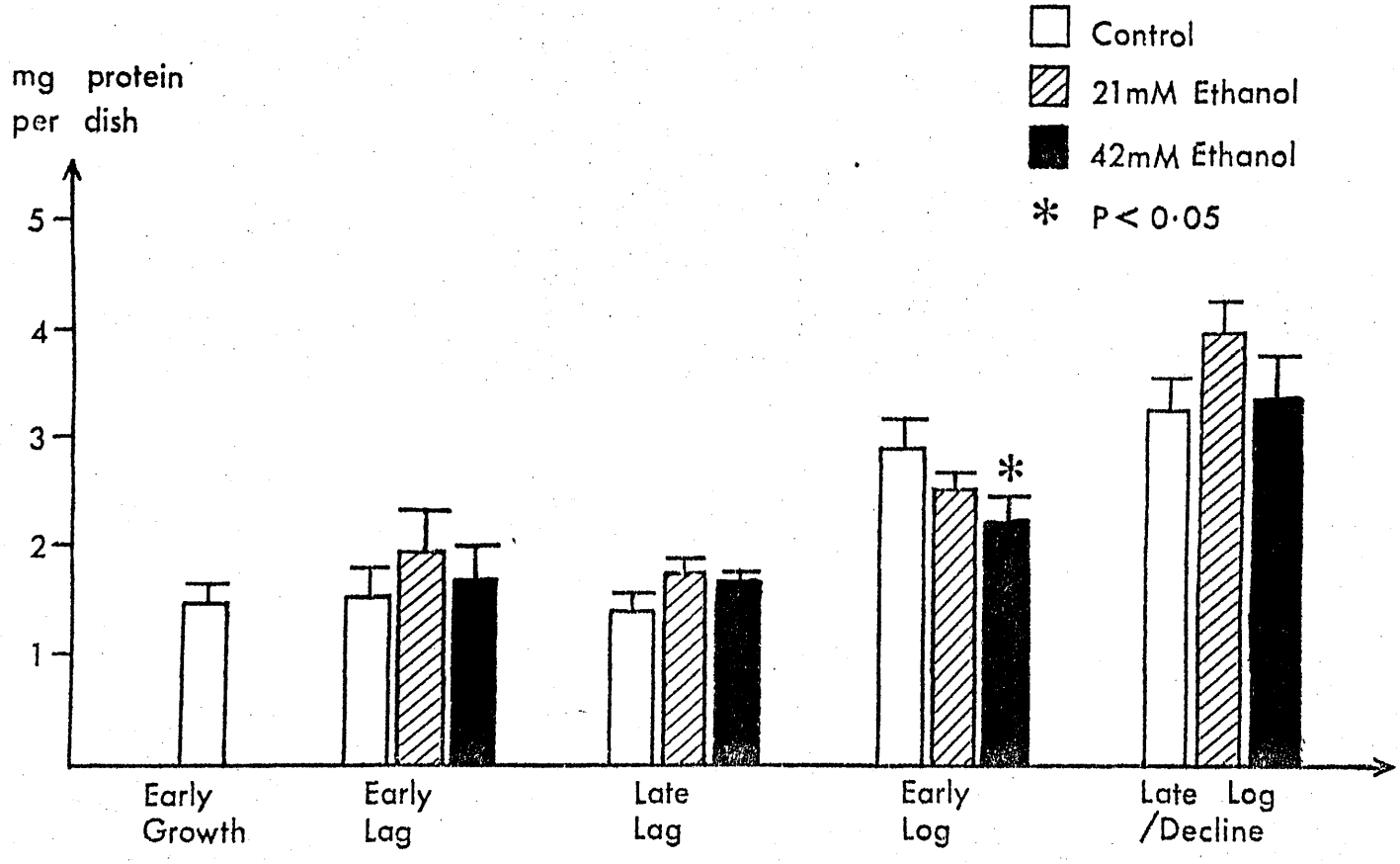
(day 6), inhibited cell division resulted in lower RNA per dish in experimental dishes (t 2.50; $p < 0.025$). The cell content of RNA tended to be lower but did not reach significance ($p > 0.05$). On days 6 and 7, when ethanol treated cells were growing logarithmically, RNA per dish increased by 110% and 155% respectively in 21mM and 42mM ethanol treated cells. In control cells the increase over each 24 hour period was 108% and 72% respectively, although the apparent increased rate of cell division in experimental cells resulted in the actual RNA content per cell tending to be lower than control cells. This finding is consistent with the division of experimental cells exhibiting a higher degree of synchrony than control.

Effects of Ethanol on Protein

During the two-day lag period of the control cells when protein was falling per dish as well as per cell, ethanol treated cells tended to have more protein per dish (Fig 28) as well as per cell (Table XII). The rise in protein per dish which occurred in control cells in the presence of cell division between days 5 and 6 (early logarithmic growth) was not as great in ethanol treated cells.

Protein per cell fell from day 6 onwards in control and ethanol treated cells. A drop in intracellular protein and rise in intracellular cyclic AMP were the only consistent events of those investigated to occur in control and experimental dishes between days 6 and 8. Furthermore cyclic AMP rose; intracellular protein fell; cell division halted; throughout the lag phase. The results suggest that changes in intracellular cyclic AMP and protein may be related to the growth state of the cells.

FIGURE 28



Effect of ethanol on culture protein at various stages of the growth period for cells plated at 2 x 10^6 cells per dish. Ethanol was added 3 days after plating, ie at the end of the early growth period. Results are mean of 10 experiments; bars indicate $\pm$ SEM.

TABLE XII

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days After Plating	PROTEIN (mg/10^6 cells $\pm$ SE)		
1	0.525±0.105	-	-
2	0.448±0.097	-	-
3	0.585±0.072	-	-
4	0.446±0.047	0.550±0.110	0.487±0.092
5	0.370±0.042	0.464±0.037	0.444±0.058
6	0.499±0.030	0.484±0.035	0.516±0.062
7	0.356±0.045	0.458±0.055	0.447±0.061
8	0.336±0.038	0.352±0.037	0.332±0.039

Intracellular protein during the growth cycle of cells plated at a density of 2 x 10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Results are average of 10 experiments.

PART II

ADDITION OF ETHANOL TO LOGARITHMICALLY GROWING CELLS

Cell Growth After Plating

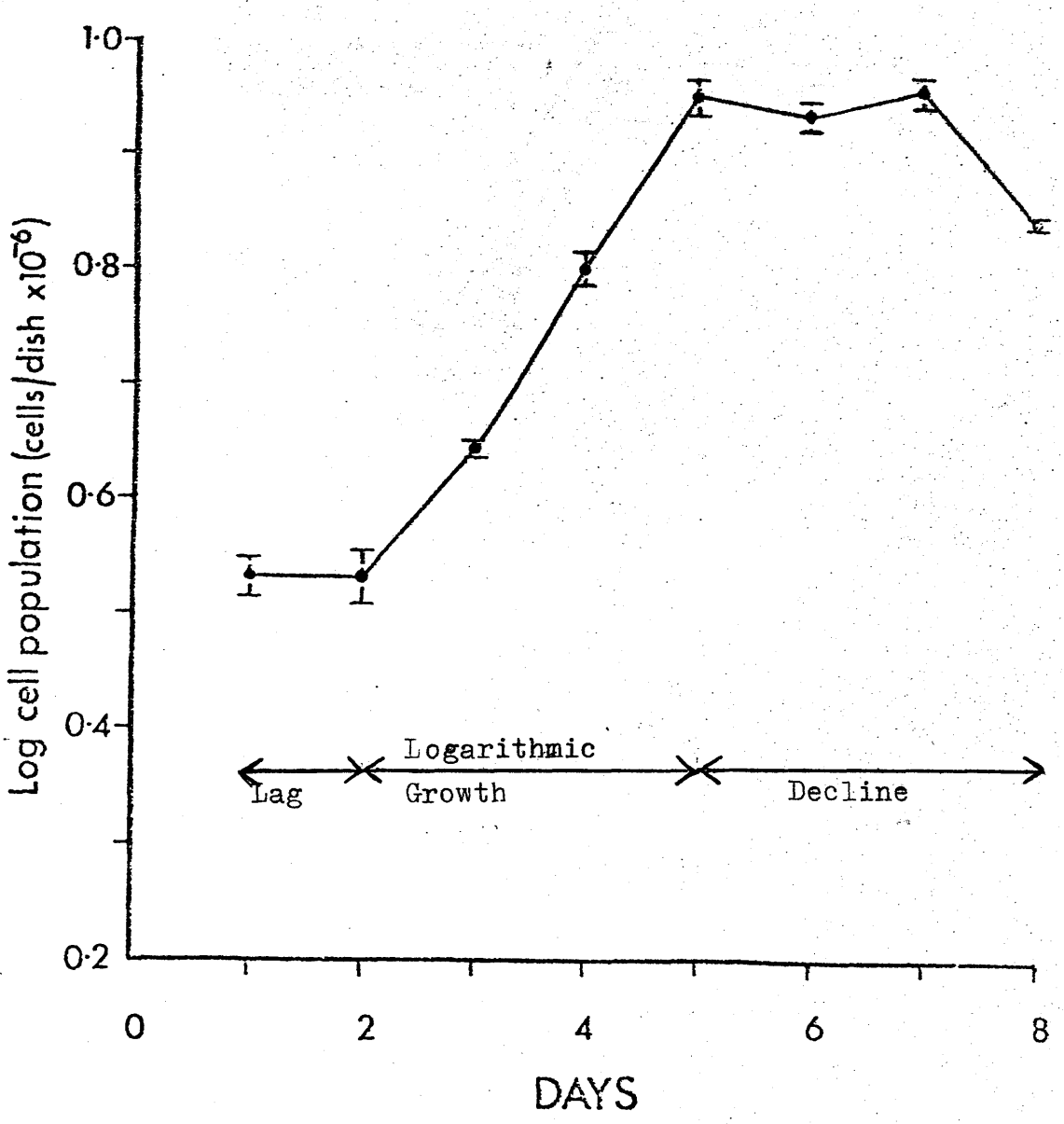
Cells plated at a density of 3.5×10^6 cells per dish began to grow logarithmically 48 hours from plating (Fig 29). The early growth period, found in the experiments quoted in Part I, was not present in this series of experiments. Logarithmic growth commenced on day 2 at a mean cell density of 3.41 ± 0.19 (SEM) cells per dish $\times 10^{-6}$ and continued till day 5. The cells tended to divide more slowly than for the previous series of experiments: population increases were 30%, 45% and 42% respectively for days 2 to 5. This result parallels the finding of Macieira-Coelho (1967) that a decreasing proportion of cells enter S phase as seeding density rises. Furthermore the growth decline phase tended to occur at a lower mean cell density (9.14 ± 0.33 cells per dish $\times 10^{-6}$) than quoted in Part I.

The growth pattern in this series of experiments is identical to that generally reported for mammalian cells (Paul 1970) and for bacterial growth (Mandelstam and McQuillen 1973).

Effect of Ethanol on Cell Population

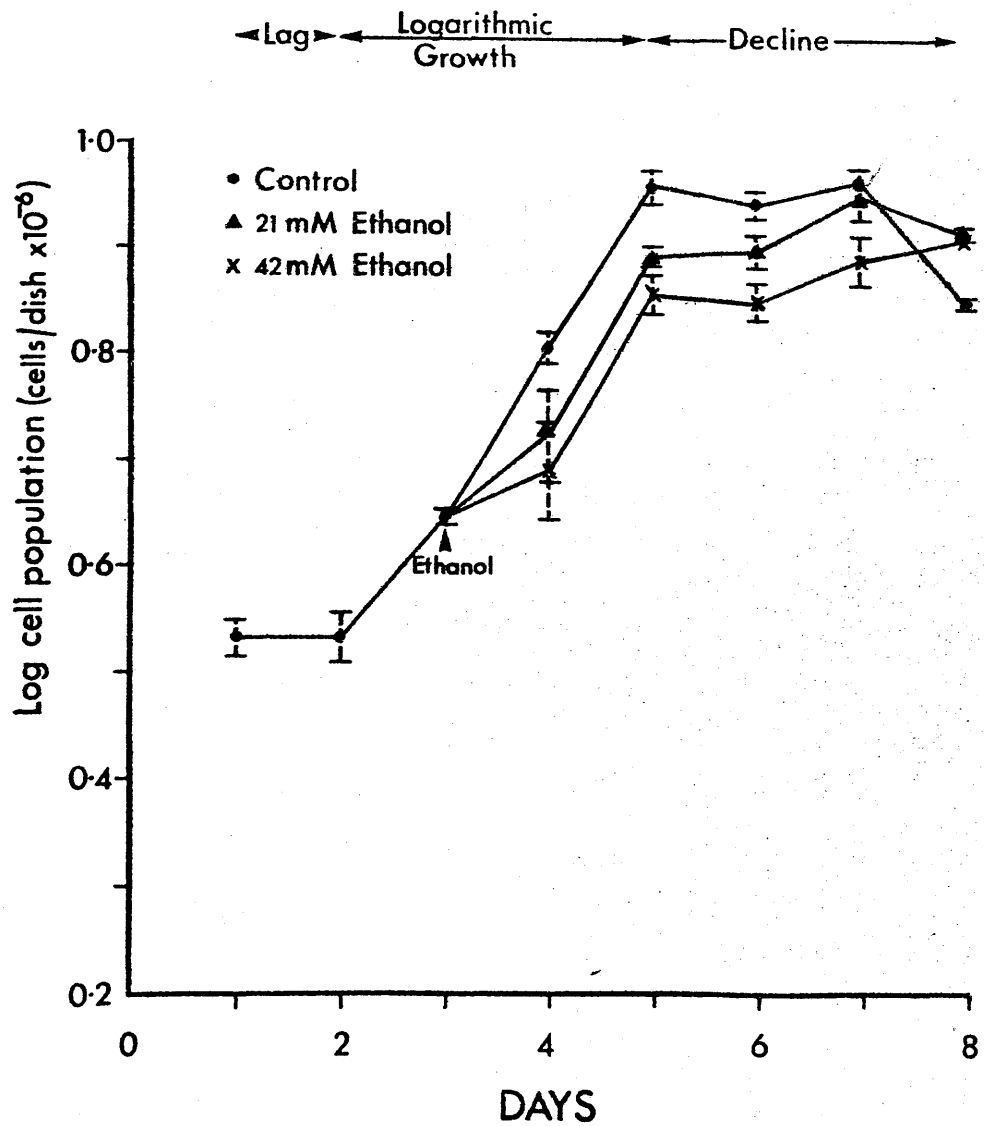
Ethanol was added at a cell density of 4.4 ± 0.1 (SEM) $\times 10^6$ cells per dish. Within 1 day of its addition (day 4), cell populations in dishes containing ethanol were significantly lower than control ($t = 2.27$; $p < 0.025$ and $t = 3.04$; $p < 0.005$ respectively for 21mM and 42mM ethanol treated cells; Fig 30). The increase in cell population between

FIGURE 29



Growth curve for L-cells plated at a cell density of 3.5×10^6 cells per dish. Points represent logarithm of the mean of 6 estimates; bars indicate $\pm$ SEM.

FIGURE 30



The effect of ethanol on cell population. Ethanol-containing growth medium was added on day 3, at a cell population of 4.4 ± 0.1 (SEM) cells per dish x 10^{-6}, when the cells were growing logarithmically. The medium was changed daily thereafter. Results are mean of 6 experiments; bars indicate $\pm$ SEM.

Cell populations in decline phase ethanol treated cells were significantly lower ($p < 0.025$) than in decline phase control cells.

days 3 and 4 was 45% in control dishes but only 20% and 12% respectively in dishes containing 21mM and 42mM ethanol. Ethanol when added to logarithmically growing cells has either decreased their rate of cell division or caused them to re-enter a lag phase. These results are similar to those obtained in the first 24 hours of logarithmic growth in the previous series of experiments.

From days 4 to 5, population increases in dishes containing 21mM and 42mM ethanol were 48% and 46% respectively. During any 24 hour period in control cells, maximum population increase was never more than 45%. As found previously, ethanol has tended to increase the growth rate of the cells, or alternatively could have caused them to divide more synchronously than control cells, during late logarithmic growth.

Timing of growth decline was the same in control and experimental dishes. Consequently, 21mM and 42mM ethanol treated cells ceased to grow logarithmically at cell densities of 7.85 ± 0.06 cells per dish x 10^{-6} and 7.22 ± 0.10 cells per dish x 10^{-6} respectively. Growth decline in control cells occurred at a cell density of 9.14 ± 0.33 cells per dish x 10^{-6}.

INTRACELLULAR CYCLIC AMP THROUGHOUT GROWTH

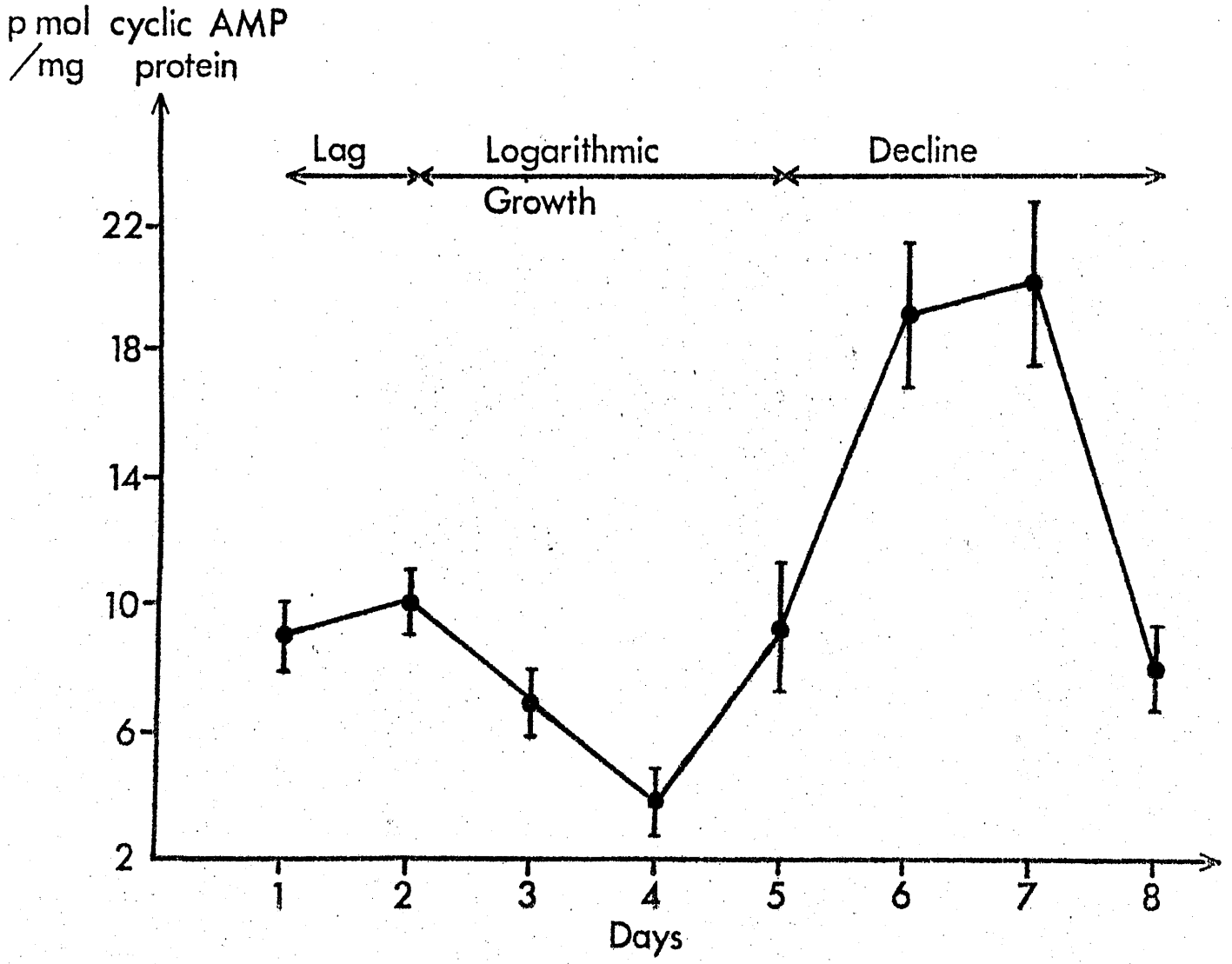
Twenty-four hours after plating, intracellular cyclic AMP concentration was 8.94±1.11 pmol/mg protein (Fig 31), which is close to the mean value obtained for early growth and early lag phase cells in the previous series of experiments. On day 2 (late lag) intracellular cyclic AMP had risen to 10.02±1.21 pmol/mg protein, ie an increase of 12%; much less than the rise of 84% (to reach 18.05 pmol/mg protein) obtained during the latter half of the lag phase in previous experiments. Thus when these cells began to grow logarithmically they had lower cyclic AMP than cells plated at the lower cell density of previous experiments.

Cyclic AMP decreased during early to mid logarithmic growth, reaching a minimum of 3.66±1.10 mg protein/10^6 cells on day 4 at a cell density of 6.42±0.23 x 10^6 cells per dish. This was lower than the concentration of 5.45±0.81 mg protein/10^6 cells found during mid logarithmic growth in previous experiments at a cell density of 6.15 x 10^6 cells/dish. Cyclic AMP per mg protein rose from mid logarithmic growth to decline (days 4 to 6) as occurred in previous experiments. A fall in intracellular cyclic AMP occurred three days after growth decline (days 7 to 8). This fall in cell cyclic AMP could account for the absence of an increase in cyclic AMP levels at optimal cell density obtained by various investigators (see review, Chlapowski et al 1975).

Effect of Ethanol on Intracellular Cyclic AMP Throughout Growth

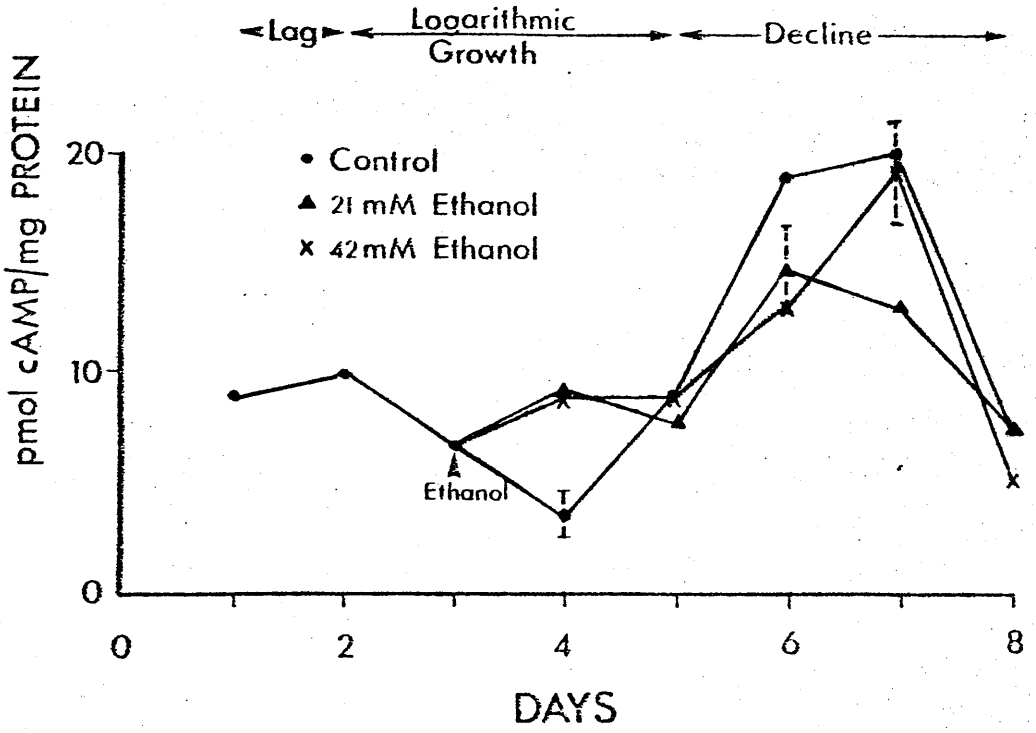
In this series of experiments, cells treated with 21mM or 42mM ethanol had elevated intracellular cyclic AMP within 24 hours of ethanol addition (Fig 32). The ethanol

FIGURE 31



Intracellular cyclic AMP, expressed as per mg intracellular protein, vs days after plating, for cells plated at 3.5×10^6 cells per dish. Each point is the mean of 4 determinations; bars indicate ± SEM.

FIGURE 32



The effect of ethanol on intracellular cyclic AMP for cells plated at 3.5×10^6 cells per dish. Ethanol containing growth medium was added on day 3 and changed daily thereafter. One day after addition of ethanol-containing growth medium (day 4) the re-entry to a lag phase in ethanol treated cells (compare Fig 30) was parallelled by increased intracellular cyclic AMP in experimental cells when compared to control.

Results are mean of 4 experiments; typical SEM bars shown.

treated cells had cyclic AMP levels on day 4 of 8.65±0.51 and 8.22±1.14 pmol cyclic AMP/10^6 cells, ie concentrations similar to those in late lag phase cells on day 2. These concentrations were more than twice the concentration in control cells. At the end of the logarithmic growth period (day 5), cyclic AMP concentrations in experimental cells were the same as control. During the growth decline phase (days 5 to 8) ethanol treated cells tended to have lower intracellular cyclic AMP than control (cf Part I).

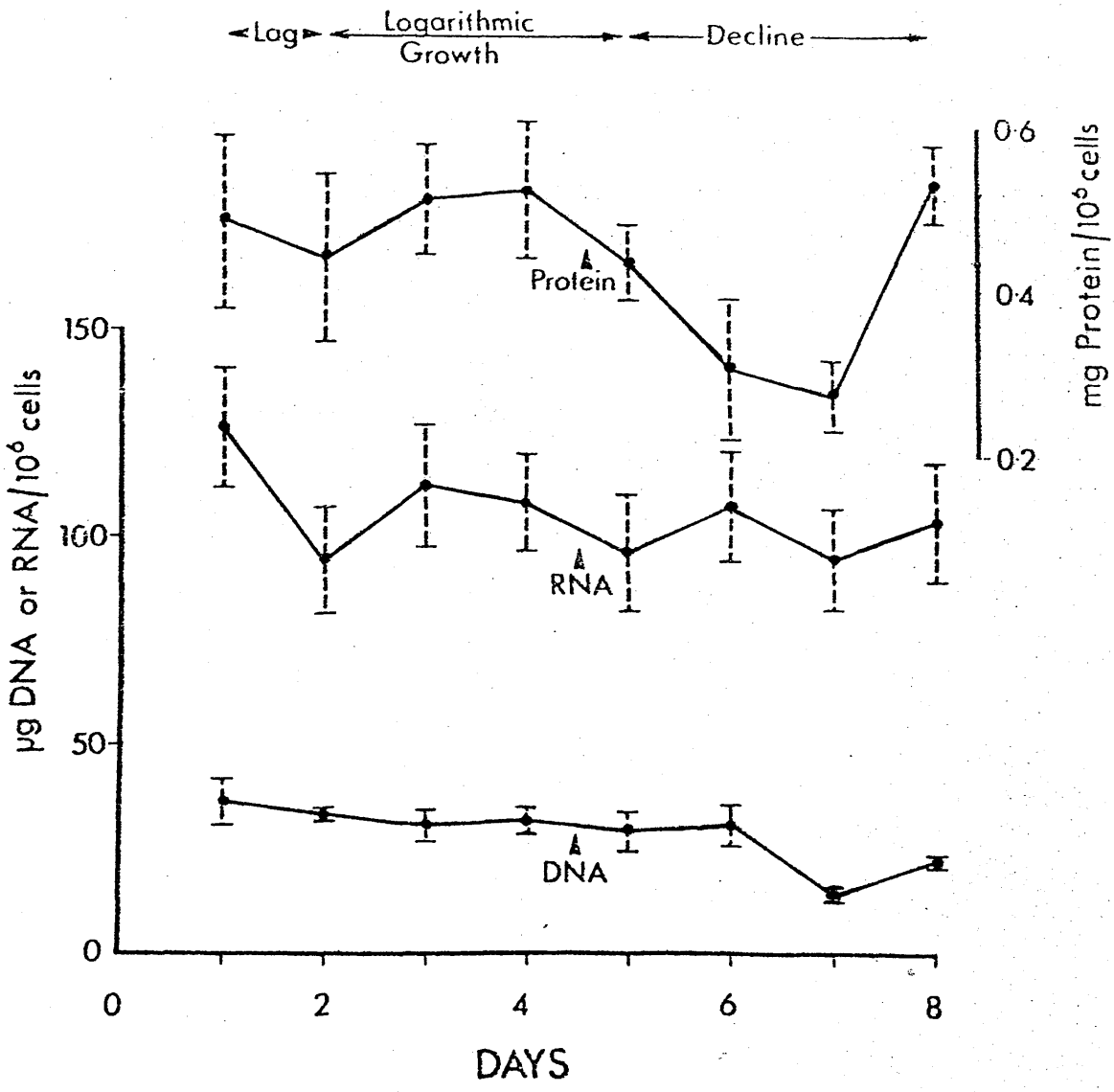
Thus the decreased growth rate, or re-entry to a lag phase, which occurred when ethanol was added to logarithmically growing cells was parallelled by increased intracellular cyclic AMP in these cells over control cells. This was found also during early to mid logarithmic growth in the previous experiments.

CHANGES IN DNA, RNA AND PROTEIN DURING GROWTH

Twenty-four hours after plating, DNA, RNA and protein content per 10^6 cells were 36.2±5.1 (SEM) µg; 126.2±15.6 µg and 0.482±0.126 mg respectively (Fig 33). For DNA and RNA, these concentrations tended to be higher than those of logarithmically growing cells. Intracellular protein measurements for day 1 were scattered (standard deviation was 47% of the mean) but were within the range of values for cells during logarithmic growth. These results imply that the cells were capable of synthesising DNA, RNA and protein during the time interval between trypsinisation and 24 hours after plating out (cf Part I). The previous series of experiments showed an early growth period with little or no synthesis of DNA, where cells in G_2 progressed through mitosis to G_1. The absence of rises in culture RNA and protein during the lag phase in these experiments (Fig 34) implies that cell division would have been less synchronous than in Part I.

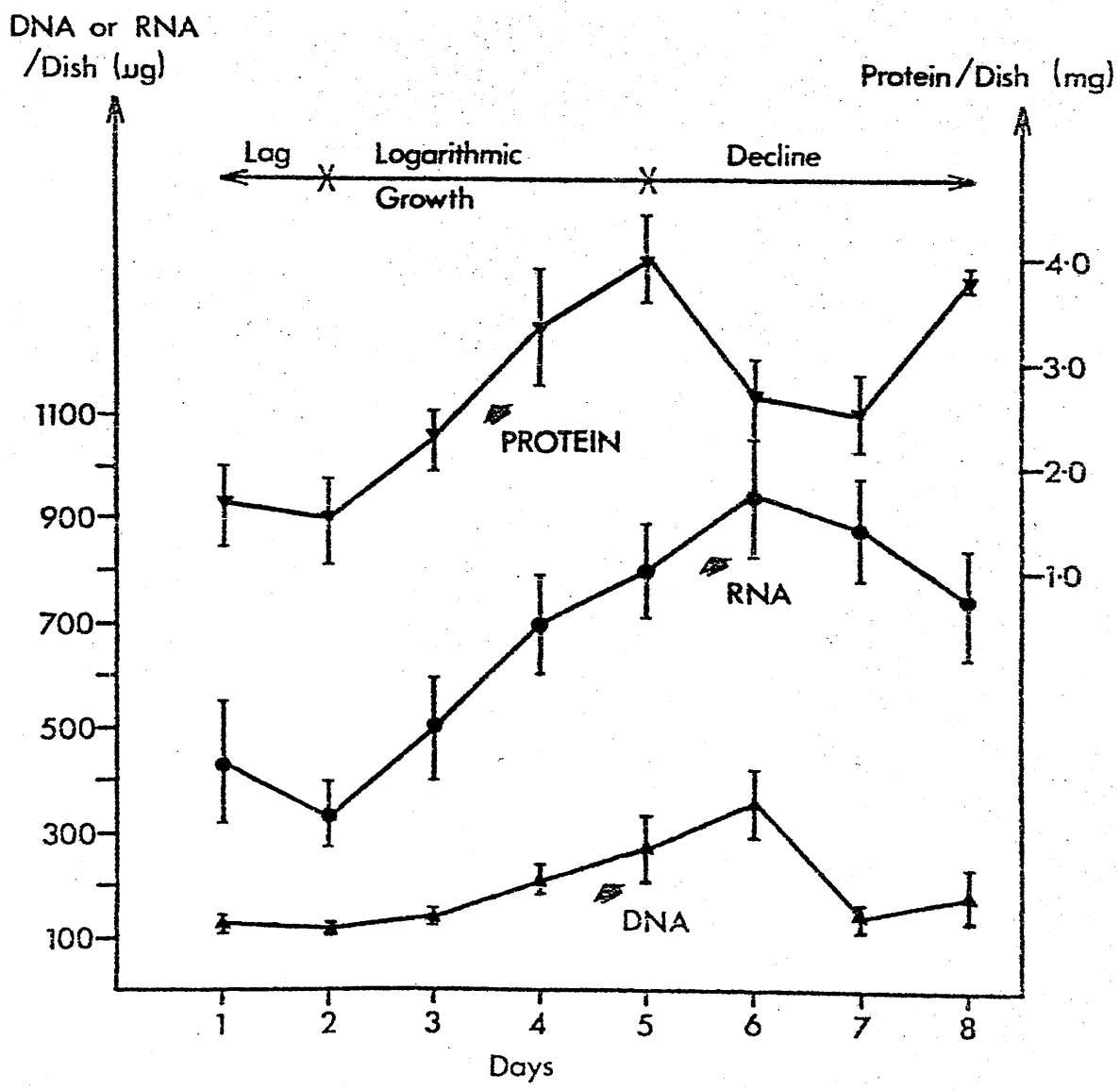
Fluctuations between mean values of cell DNA, RNA and protein from day to day were less than for previous experiments (compare Figs 33 and 24). Furthermore absolute levels tended to be higher, eg at logarithmic growth in present experiments, intracellular DNA, RNA and protein were 32.1±3.1 µg; 108.7±12.3 µg and 0.523±0.092 mg per 10^6 cells respectively, at a cell density of 6.42±0.23 cells per dish x 10^{-6} whereas in the previous experiments (day 6), concentrations were 28.8±1.4 µg; 95.6±11.8 µg and 0.474±0.030 mg per 10^6 cells respectively at a cell density of 6.15±0.22 cells per dish x 10^{-6}. Consequently, cell DNA did not increase progressively during

FIGURE 33



Intracellular DNA, RNA and protein from 1 to 8 days after plating, for L-cells plated at 3.5×10^6. Measurements were obtained on washed cells, as described in Methods. Each point represents the mean of 6 measurements; bars indicate $\pm$ SEM.

FIGURE 34



Total culture DNA, RNA and protein from 1 to 8 days after plating, for L-cells plated at 3.5 x 10^6 cells per dish. Estimates were calculated on measurements of intracellular DNA, RNA and protein of washed cells, obtained as described in Methods, multiplied by the cell population per dish. Each point represents the mean of 6 estimations; bars indicate $\pm$ SEM.

logarithmic growth. This finding is in contrast to that found in previous experiments (Fig 24). In the experiments outlined in Part I RNA per cell rose throughout logarithmic growth, in the present experiments RNA fell from mid to late logarithmic growth. Furthermore in present experiments, an increase in RNA did not preceed an increase in DNA before commencement of logarithmic growth. Whereas the previous experiments showed that RNA synthesis appeared largely independent of DNA synthesis during early growth and lag phases, results of present experiments imply that RNA synthesis is dependent on DNA synthesis during logarithmic growth.

Intracellular protein began to fall on day 4 (late logarithmic growth). On day 5, protein content began to decline in plates despite continued increases in RNA and DNA per dish. The results suggest that a decrease in protein may determine the timing of growth decline.

Intracellular Cyclic AMP in Relation to DNA, RNA and Protein

In Part I, intracellular cyclic AMP rose by 84% during the 24 hours before commencement of logarithmic growth. As described above, cyclic AMP rose by only 12% in the current experiments. Such a finding may be related to the higher cellular protein, RNA and DNA in present experiments. Alternatively the "peak" of cellular cyclic AMP obtained in previous experiments could have been obscured in present ones by virtue of more asynchronous growth in the absence of an early growth phase. In both series of experiments a drop in cell cyclic AMP was concurrent with increased DNA, RNA and protein per plate, and cell division, as logarithmic growth commenced. Furthermore elevations in intracellular cyclic AMP and decreases

in intracellular protein appear to be involved in growth decline.

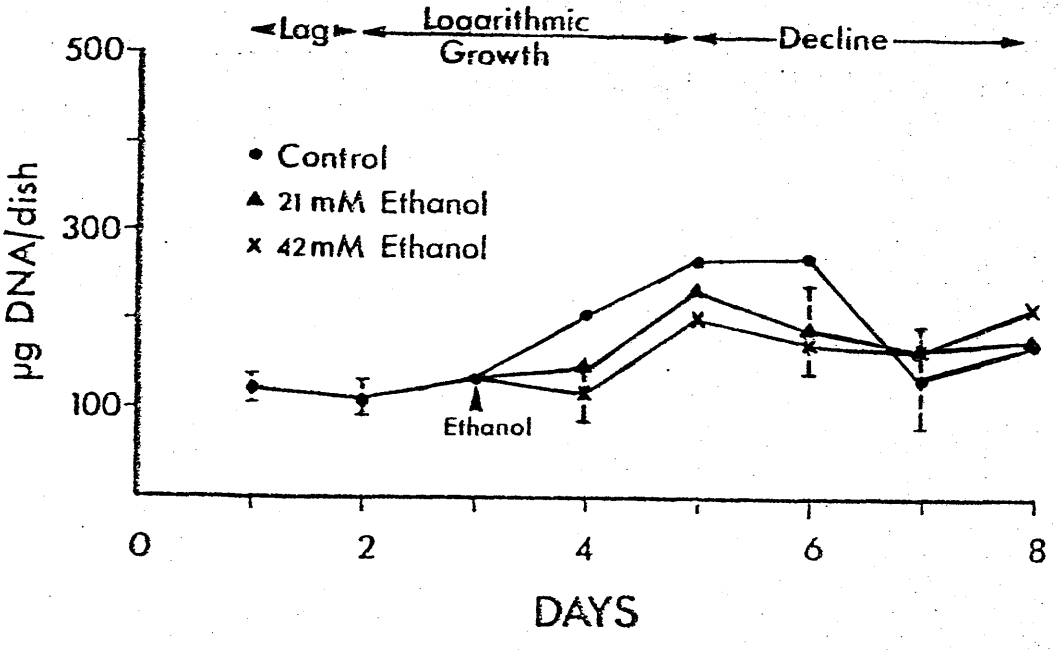
Effect of Ethanol on DNA

As before, addition of ethanol resulted in a decreased DNA content per plate within 24 hours of its addition (Fig 35). DNA content per cell on day 4 was lower in experimental cells (Table XIII). Between days 4 and 5, DNA content per dish rose by 60% and 74% respectively in 21mM and 42mM ethanol treated cells. Increase in DNA for control dishes over any 24 hour period was never more than 53%. This result is consistent with the growth rate of ethanol treated cells being higher during logarithmic growth than that of control cells. The result is also consistent with the cells growing more synchronously. Ethanol treated cells had lower intracellular DNA than control cells for the same cell density (both series of experiments; Fig 36).

Effects of Ethanol on RNA

Within 24 hours of ethanol addition (day 4) inhibited cell division had resulted in lower RNA content per dish in treated cells (Fig 37). RNA content per cell also tended to be lower in experimental cells (Table XIV). (This effect occurred at early logarithmic growth in the previous experiments). Ethanol potentiated the fall in RNA per cell which occurred from mid to late logarithmic growth. Consequently, as cell numbers rose in experimental and control cells, RNA per dish increased by 18% and 21% respectively in 21mM and 42mM ethanol treated cells. Percentage rise in control cells was never less than 26% during logarithmic growth. This effect is opposite to the relative

FIGURE 35



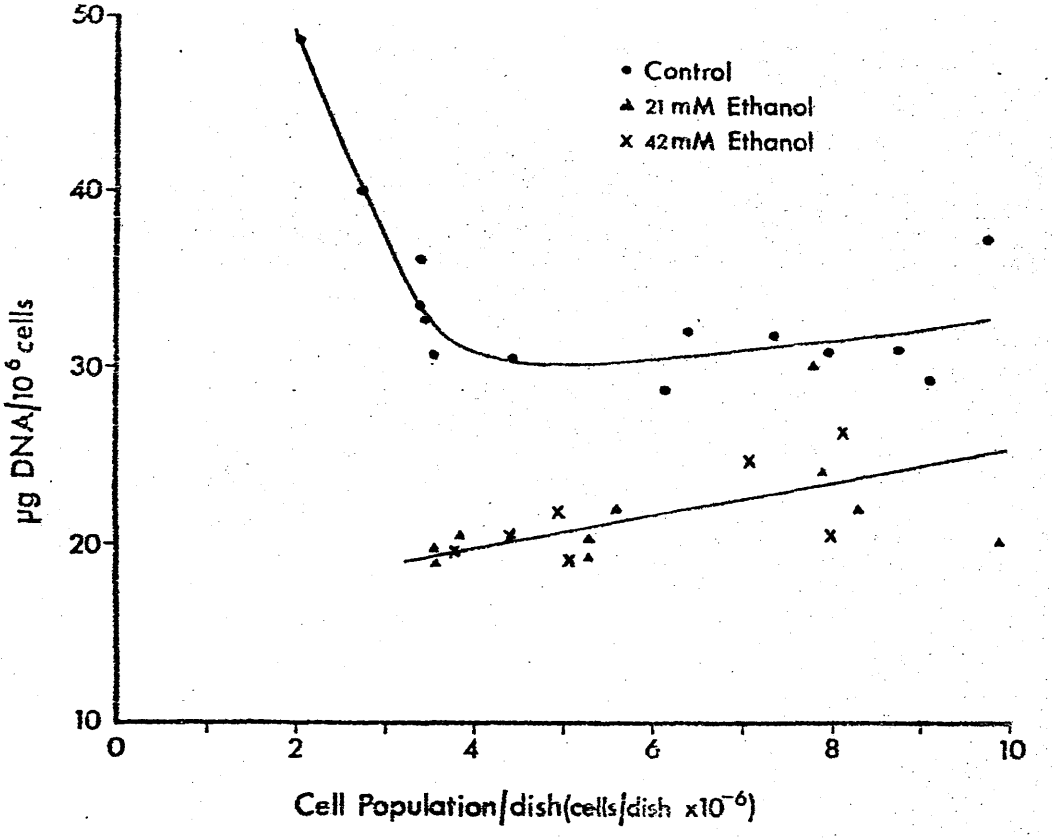
The effect of ethanol on culture DNA for cells plated at 3.5×10^6 cells per dish. Ethanol-containing growth medium was added on day 3 and changed daily thereafter. Results are mean of 4 experiments; typical SEM bars shown.

TABLE XIII

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days after Plating	DNA ($\mu g/10^6$ cells $\pm$ SE)		
1	36.2 ± 5.1	-	-
2	33.5 ± 1.3	-	-
3	30.5 ± 3.7	-	-
4	32.1 ± 3.1	27.8 ± 2.2	23.6 ± 4.6
5	29.4 ± 4.6	30.1 ± 4.8	28.1 ± 5.6
6	31.1 ± 4.8	24.1 ± 6.1	24.7 ± 5.7
7	14.6 ± 2.0	18.7 ± 4.5	20.6 ± 5.1
8	22.5 ± 1.6	21.9 ± 4.6	26.4 ± 4.6

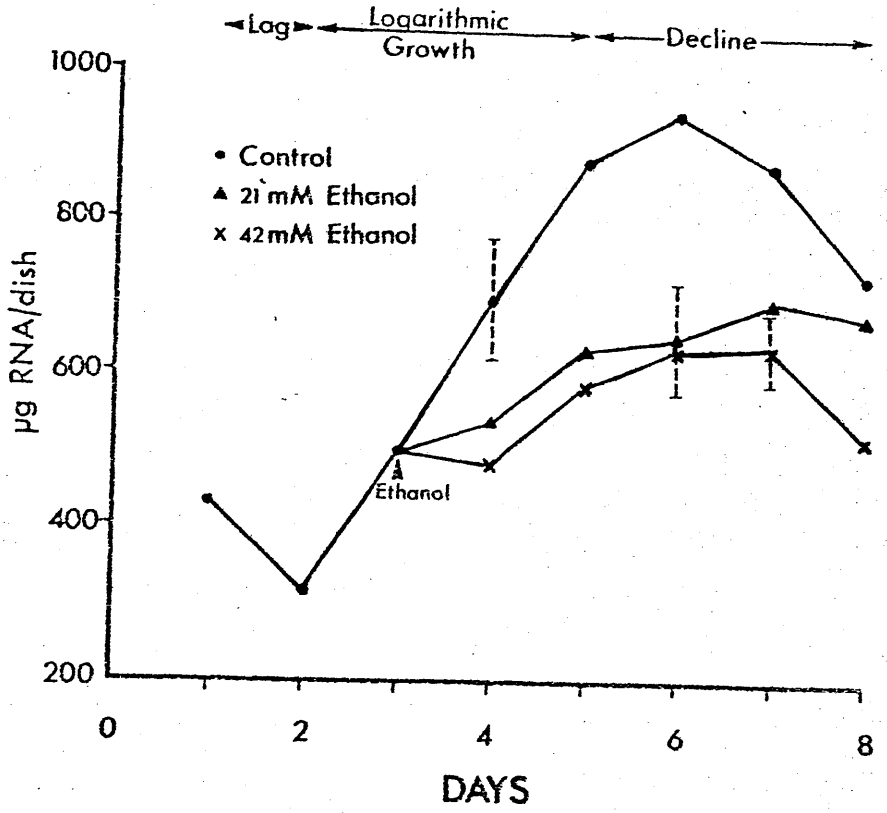
Intracellular DNA during the growth cycle of cells plated at a density of 3.5×10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Growth medium was changed daily from day 3 onwards in all dishes, as described in Methods. Results are average of 4 experiments.

FIGURE 36



Intracellular DNA vs cell density for control and experimental dishes. Lines drawn by eye.

FIGURE 37



The effect of ethanol on culture RNA for cells plated at 3.5×10^6 cells per dish. Ethanol-containing growth medium was added on day 3 and changed daily thereafter. Results are mean of 4 experiments; typical SEM bars shown.

TABLE XIV

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days after Plating	RNA/μg(10^6 cells $\pm$ SE)		
1	126.2$\pm$15.6	-	-
2	94.2$\pm$13.8	-	-
3	112.7$\pm$16.2	-	-
4	108.7$\pm$12.3	101.3$\pm$14.6	98.4$\pm$14.3
5	96.6$\pm$15.2	80.6$\pm$8.7	81.3$\pm$15.1
6	107.7$\pm$15.1	80.6$\pm$9.1	89.4$\pm$12.0
7	95.0$\pm$12.1	77.6$\pm$7.6	81.9$\pm$15.0
8	104.0$\pm$15.3	82.0$\pm$15.6	63.6$\pm$15.5

Intracellular RNA during the growth cycle of cells plated at a density of 3.5 x 10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Results are average of 4 experiments.

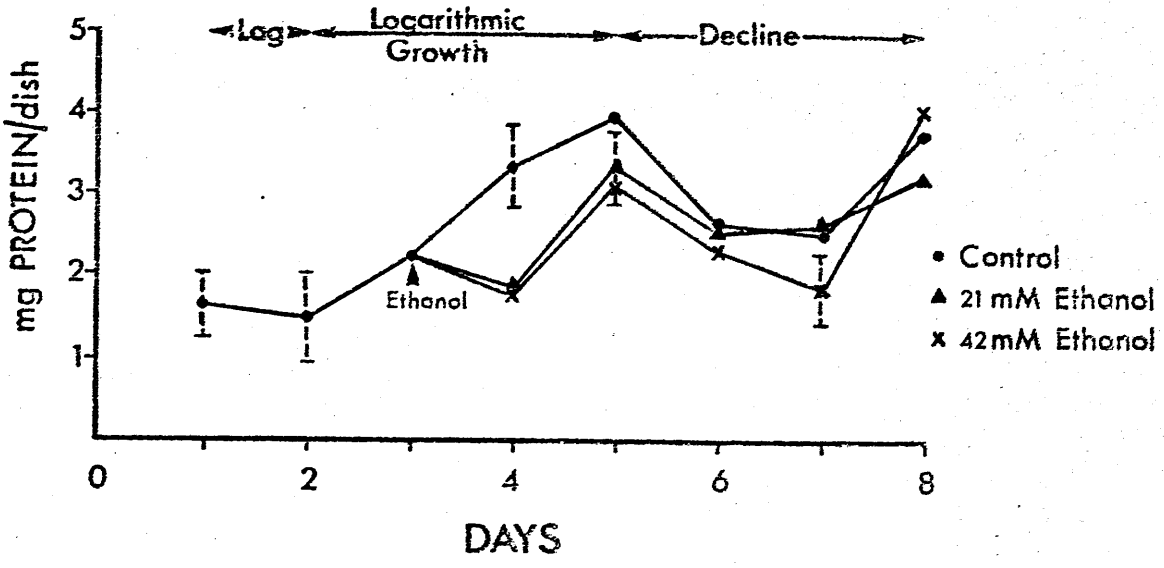
increases in RNA in control and experimental cells from mid to late logarithmic growth (days 6 to 7) in previous experiments, where RNA per cell was rising.

Effects of Ethanol on Protein

Protein per dish in 21mM and 42mM ethanol treated cells increased by 79% and 76% respectively between days 4 and 5 (Fig 38). Percentage rise in control cells was never more than 52% over any 24 hour logarithmic growth period. Protein per cell also rose between days 4 and 5 in experimental dishes, to reach the same protein content as control cells on day 5. (Table XV).

On day 5, intracellular cyclic AMP values for control and experimental dishes were not significantly different (Fig 32) in the presence of similar protein (Table XV) intracellular DNA (Table XIII) but lowered intracellular RNA (Table XIV) and cell numbers. From day 5 onwards, protein per dish declined in experimental dishes as well as in control dishes. Intracellular protein tended to be higher in decline phase cells of ethanol treated cells compared to control. Cyclic AMP tended to be lower. Intracellular protein for pooled results (control and experimental; both series of experiments) showed a negative log linear relationship with picomoles cyclic AMP per milligram protein over the range 0.25 to 0.60 mg protein per 10^6 cells ($r = -0.77$; Fig 39).

FIGURE 38



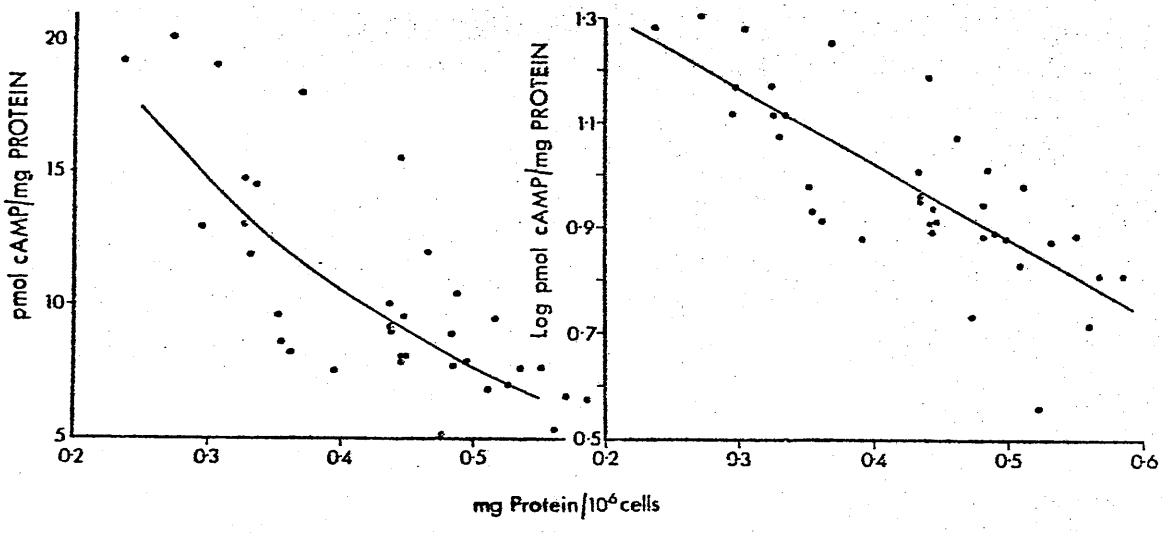
The effect of ethanol on culture protein for cells plated at 3.5×10^6 cells per dish. Ethanol-containing growth medium was added to experimental cells on day 3 and changed daily thereafter. Results are mean of 4 experiments; typical SEM bars shown.

TABLE XV

Growth Medium	Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Days after Plating	PROTEIN (mg/10^6 cells $\pm$ SE)		
1	0.482±0.126	-	-
2	0.437±0.168	-	-
3	0.510±0.066	-	-
4	0.523±0.0092	0.355±0.051	0.362±0.048
5	0.436±0.047	0.429±0.044	0.436±0.047
6	0.306±0.102	0.326±0.063	0.327±0.094
7	0.273±0.045	0.294±0.049	0.238±0.036
8	0.533±0.047	0.393±0.055	0.561±0.092

Intracellular protein during the growth cycle of cells plated at a density of 3.5 x 10^6 cells/dish. In experimental dishes, ethanol containing medium was added on day 3. Results are average of 4 experiments.

FIGURE 39



(i) (ii)

Relationship of intracellular cyclic AMP to intracellular protein. Points represent results from control and experimental cells in both series of experiments. (Parts I and II of Results). For graph (ii), r =-0.77 for the range 0.2 to 0.6 mg protein/10^6 cells.

SUMMARY OF EFFECTS OF ETHANOL

(i) Ethanol had no effect on cell numbers during the lag phase, when the cells were not actively dividing.

(ii) Cyclic AMP rose sharply during the latter 24 hours of the lag phase. Ethanol addition blunted this rise in cyclic AMP.

(iii) Ethanol significantly decreased intracellular DNA content during lag and logarithmic growth phases. Ethanol treated cells had lower DNA than the same cell density in control cells.

(iv) RNA rose in the absence of a rise in DNA during the latter half of the lag phase. Ethanol did not prevent, but tended to decrease the rise in RNA.

(v) Ethanol had no significant effect on protein content of cells when they were not actively dividing (lag phase). However, protein per cell tended to be higher in ethanol treated cells.

(vi) During early-mid logarithmic growth in control cells, ethanol either

(a) decreased growth rate, or

(b) potentiated or initiated a lag phase.

Concurrent with inhibited cell division there occurred a decreased intracellular DNA and an increased intracellular cyclic AMP.

(vii) Ethanol potentiated the fall in RNA per cell when added to logarithmically growing cells. This was parallelled by decreased intracellular DNA and protein and increased intracellular cyclic AMP.

(viii) At the most rapid 24 hour growth period in ethanol-

treated cells, rate of increase of cell population and protein per dish was greater than over either 24 hour logarithmic growth period in control cells. Cells tended to be poor in content of RNA and DNA.

(ix) Ethanol did not affect the timing of growth decline. A drop in intracellular protein and simultaneous rise in intracellular cyclic AMP were the only consistent events of those investigated to occur in the decline phase in control and experimental dishes. Elevations in intracellular cyclic AMP and decreases in intracellular protein appear to be involved in decline phase and lag phase growth.

PART III

TIME COURSE OF CHANGES IN CELL CYCLIC AMP IN RESPONSE TO ETHANOL

Short term experiments were carried out to determine the time length of any changes in intracellular cyclic AMP produced by ethanol. The experiment was carried out in serum free medium: in several cell lines, the presence of serum has been reported to lowel basal cyclic AMP levels (see Introduction) and to reduce the magnitude of the cyclic AMP responses to catecholamines and prostaglandins (Makman et al 1974). Cell densities used for experiment corresponded to early growth, early lag and logarithmic growth phases of the growth period.

Table XVI shows that ethanol addition resulted in an increase in intracellular cyclic AMP. In some cases, the effect became evident within 1 minute. However, the effect was not always consistent throughout the time intervals measured; it did not vary in any consistent manner with time or with concentration of ethanol. The elevations in cell cyclic AMP produced are presumed to be a non-specific response. Sheppard (1972b) has postulated that elevations in cellular cyclic AMP levels are most likely a response to extremely poor growth conditions. It has already been mentioned (page 18) that depletion of serum factors in cultured fibroblasts is reflected by a rise of intracellular cyclic AMP. An additional response became evident in these experiments, that is, that at the 1 hour time interval of incubation, cellular cyclic AMP in control cells tended to be elevated. This lends more weight to the reports that serum starvation results in increased intracellular cyclic AMP.

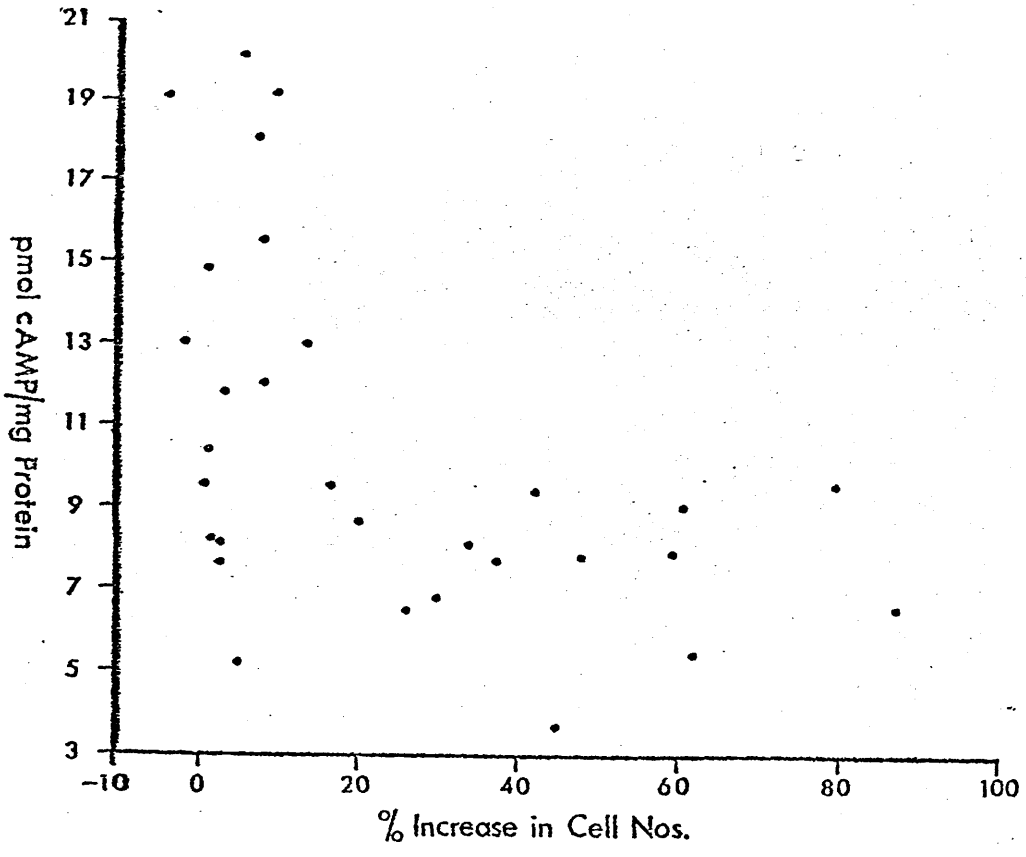
TABLE XVI

Serum-free Growth Medium Added		Zero Ethanol (Control)	21mM Ethanol	42mM Ethanol
Cell Density (cells/dish x 10^{-6})	Time of Incubation (mins)	pmol cyclic AMP/10^6 cells $\pm$SE		
1.48 (early growth)	1	1.78$\pm$0.35	8.82$\pm$0.32	1.14$\pm$0.31
	5	1.22$\pm$0.18	2.62$\pm$0.78	8.03$\pm$0.92
	15	2.42$\pm$0.30	7.80$\pm$0.96	6.29$\pm$1.36
	60	2.46$\pm$0.38	8.13$\pm$0.30	8.83$\pm$0.91
2.82 (lag)	1	3.41$\pm$1.11	2.23$\pm$0.46	6.76$\pm$0.26
	5	2.68$\pm$1.62	3.96$\pm$1.84	4.06$\pm$1.76
	15	2.62$\pm$0.53	2.44$\pm$0.47	5.03$\pm$0.65
	60	5.81$\pm$1.10	5.67$\pm$1.43	4.99$\pm$0.63
8.03 (logarithmic growth)	1	3.03$\pm$0.46	2.91$\pm$0.59	2.48$\pm$0.11
	5	4.08$\pm$0.15	4.02$\pm$1.09	5.07$\pm$1.26

Short-term effect of ethanol on intracellular cyclic AMP during early growth, early lag and logarithmic phases of the growth cycle. Dishes had been equilibrated for 2 hrs in serum-free medium at room temperature before initiation of experimental conditions. The experiment was carried out at room temperature. For each estimation n=3.

Cell cyclic AMP was not related in any direct manner to the growth rate of the cells over the day of its measurement (Fig 40).

FIGURE 40



Relationship of intracellular cyclic AMP to the growth rate of the cells over the day of its measurement. Points represent results from control and experimental cells in both series of experiments (Parts I and II of Results).

For the range -5% to 100% increase in cell numbers, $r = 0.50$.

DISCUSSION

PRIMARY ASSUMPTIONS ON THE GROWTH STATE OF THE CELLS

The purpose of this section is to discuss some intracellular processes involved in the growth of L929 cells in culture. Several hypotheses will be advanced in an attempt to explain the changes found on incubation with alcohol.

The original aim of the work was to examine the sequence of events occurring when resting (decline phase) L929 cells are induced to divide and to study the effects of ethanol on growth control in the cell line. All cells used for experiments were subcultured from parent cultures which had been grown to high population densities and were thus in the decline phase of growth. Cells obtained commercially were transported in refrigerated containers. Consequently, several major features of cell culture populations which had previously been reported in the literature could be assumed:

(i) High population density decline phase cultures are arrested in the G_1 phase of the cell cycle. Such populations, when subcultured into fresh medium, will have conferred upon them a degree of synchrony as a result of delayed entry into S phase (Lindsay 1969).

(ii) Further cooling of cells to $4^{o}C$ confers upon them a degree of synchrony (depending on the time length of the incubation at $4^{o}C$) by preventing entry into S phase (Newton and Wildy 1959; Cameron and Padilla 1966).

GROWTH OF L-CELLS FOLLOWING SUBCULTURE

Growth Rate as a Function of Plating Density

In the first series of experiments, cells were plated at 2×10^6 cells per dish. This corresponds to a cell density of 2.6×10^4 cells/cm^2 (using a radius of 5cm when calculating the surface area of the dish). Confluency, which is the completion of contact between opposed cell borders, has been reported using various cell lines as occurring at a cell density of 4 to 6 $\times 10^4$ cells/cm^2 (eg Schutz and Mora 1968; Pontén et al 1969; Bannai and Sheppard 1974). Thus the plating density in the first series of experiments corresponds to an incomplete monolayer and cell-cell contact would not have been complete. In the second series of experiments, the cells were plated at a cell density of 3.5×10^6 cells per dish (4.5×10^4 cells/cm^2), which would have resulted in the formation of an almost complete or complete monolayer and cell-cell contact without cell division.

Skehan and Friedman (1974) found that L-cells plated at a sparse cell density (1.4 to 3.6 $\times 10^3$ cells/cm^2) showed an initial period of growth rate acceleration which was dependent upon plating density. The initial growth rate (from 24 to 48 hours after plating) was proportional to the initial seeding density. As their cultures approached confluency however, the growth rate decellerated to a low but non-zero value; thus a maximum growth rate was achieved at some point during this pre-confluent growth period. They interpreted their findings to mean that co-operative growth-facilitating reactions occur between cells, which require either intercellular contact or a high degree of proximity. They proposed that such a mechanism

of growth control allows a population density to be achieved which affords a reasonable survival probability. Such co-operative growth facilitation has been noted by others (Puck and Marcus 1955; Rein and Rubin 1968). By analogy with the hypothesis of Skehan and Friedman (1974) the purpose of the early growth period in my experiments was to allow the culture a reasonable survival probability.

Timing of the Lag Phase as a Function of Plating Density

Cessation of growth upon confluency in cell lines subject to density dependent inhibition of growth has been reported as occurring at cell densities of: 5×10^4 cells/cm^2 (3T3; Todaro et al 1964; 1965); 4×10^4 cells/cm^2 (mouse embryo fibroblasts; Schutz and Mora 1968); 6×10^4 cells/cm^2 (human glia-like cells; Westermark 1971). The mechanism of the temporary decrease in rate of cell division occurring at the lag phase in Part I of the results is likely to be analagous to "contact" inhibition of cell growth at confluency in primary cell lines.

Further investigations by Skehan (1976) on the period of growth rate acceleration mentioned above, revealed that the amplitude of the maximum growth rate achieved during the pre-confluent period decreased with increasing seeding density. The timing of this peak in growth rate was independent of seeding density: hence time taken to reach confluency fell as seeding density increased. My results are in agreement with those of Skehan (1976) since the time taken to reach the logarithmic phase of growth fell as seeding density increased (Fig 18).

Timing of the Growth Decline Phase

The growth decline phase following logarithmic growth commenced at mean cell densities of 12.5×10^4 and 11.6×10^4

cells/cm^2 respectively in the two series of experiments. These cell densities still correspond to a monolayer, presumably with considerable overlap among the cells (Kruse et al 1969). Growth decline at these cell densities may have been due to the lack of adequate substratum for attachment (Van Scott and Flaxman 1968; Dulbecco and Elkington 1973) or inadequate nutrition (Castor 1970; Martin et al 1971). Alternatively, it may have been due to the endogenous mechanism of growth control proposed by Skehan (1976) which he stated as occurring also in multilayered cultures.

Growth did not stop at these cell densities, since the few cells which became detached at medium changes were replaced by new cells inserting into the monolayer (evident in Fig 30).

The Intervention of Ethanol in Cell Division

The decreased production of cells after ethanol treatment, when logarithmic growth was taking place in control cells, could have been caused by:

(i) an extended cell cycle

(ii) an increase in the proportion of non-dividing cells eg, cell death

(iii) cell detachment.

Since ethanol showed no evidence of affecting cell adhesion when compared to control plates, possibility (iii) would be unlikely. The first two possibilities ultimately mean some alteration in the control of cell division (Baserga 1965). Results from cell counts alone give no indication of where and how ethanol intervened in the cell cycle.

Ethanol extended the time length of the lag phase but did not

affect the timing of the growth decline phase, perhaps favouring the hypothesis of Skehan (1976) that some endogenous mechanism determines the timing of the growth decline phase.

THE CYLCIC AMP ASSAY: METHODOLOGICAL PROBLEMS

Reliability of the Assay

The reliability of any protein binding assay depends on the degree of fulfillment of the four parameters: specificity, accuracy, precision, and sensitivity (Rees Midgeley et al 1969; Ekins 1970) throughout the range of concentrations for which it is to be used. In the Methods section, I reported on some of the practical problems encountered in establishing a valid assay for measurement of cyclic AMP in tissue culture cells grown as a monolayer.

Tris/EDTA Buffer and Hydrochloric Acid Extraction

These methods of extraction of intracellular cyclic AMP were found to give spurious results due to inadequately rapid denaturation of the enzymes of cyclic AMP metabolism. This difficulty has been described by Otten et al (1972) who advise against any rinsing of the cell sheet between the removal of the medium and the addition of the extraction agent.

Trichloroacetate and Perchlorate Extraction

These reagents ,commonly used as deproteinising agents for extracting cyclic AMP from biological media, when incompletely removed from cell extracts gave rise to non-specific effects which were not uniform throughout the standard curve. This observation invalidates the addition of phosphodiesterase to samples by investigators (eg Otten et al 1972; Rudland et al 1974b), the remaining assayable material being subtracted as a quantitative measure of non-specificity. The magnitude of the correction made would have to depend on the concentration

of nucleotide in the system. In addition, phosphodiesterase was found to decrease the precision of the results by virtue of the procedure used in its addition and subsequent denaturation, (Fig 12). Similarly, addition of an internal standard (eg Brown et al 1971) is not a reliable indicator of non-specificity unless a complete standard curve is carried out for each sample (Weller et al 1972). Thus although non-specific interference in protein binding assays for cyclic AMP is well documented (Albano et al 1974; Bronstrom and Kon 1974; Arner and Östman 1975) standard methods of "correcting" for such non-specific effects are not always valid. The results imply that a significant cause of inaccurate cyclic AMP determinations would tend to stem from interfering substances resulting from the extraction procedures employed.

Reliability of the Method Used

The method which was established involved extraction using 5% trichloracetic acid followed by ether washing and further purification using ion-exchange chromatography. This procedure yielded measurements with adequate specificity. Otten et al (1972) reported interference from Dowex 50W x 8 resin, not found in my laboratory, where carefully washed analytical grade resin was used. The method is capable of determining cyclic AMP concentrations with an accuracy of no less than 97.3% and a minimum precision of within 17.5% for the experimental range of standards used, ie 0.5 to 4 pmol per 50 µl sample. Precision could be improved (approaching 11% quoted by the manufacturers) by the inclusion of a standard curve at the beginning, middle and end of an assay.

INTRACELLULAR CYCLIC AMP

There were considerable variations in cellular cyclic AMP content during the various stages of the growth period from early growth to decline (mean values fluctuated from a minimum of 3.66 pmol/mg protein at logarithmic growth phase to a maximum of 20.21 pmol/mg protein at decline phase. An important aspect involved in quantitatively comparing cyclic AMP results between investigations is a simultaneous comparison of population densities. As far as I can find there are no reports which relate intracellular cyclic AMP levels to cell densities over the entire period of growth from pre to post confluency. Diversity of interests, approaches, cell lines, technical details and terminology have combined to complicate the study of cyclic AMP metabolism in cultured cells (see review, Chlapowski et al 1975).

Quantitative Comparisons with Other Investigators

Heidrick and Ryan (1971) reported no measurable quantities of cyclic AMP in L929 cells until development of a stationary population at 19.2 x 10^4 cells/cm^2. The decline phase commenced in my experiments at mean cell densities of 12.5 and 11.6 x 10^4 cells/cm^2 respectively in control cells for the two series of experiments. In terms of cell density, their results are comparable to mine only for late logarithmic growth/decline cells. Although their cyclic AMP measurements qualitatively parallel mine, their values of 10.7 to 13.9 pmol per 10^6 cells on confluency are more than twice those obtained in these experiments. The finding that they obtained no measurable quantities of cyclic AMP during

the two days previous to their growth decline period, when cellular cyclic AMP levels were lower, implies that their assay method was comparatively insensitive. According to the nature of the non-specific effects in the assay of cyclic AMP in my investigations and those of others (Albano et al 1974: Brostrom and Kon 1974; Arner and Östman 1975), high levels of cyclic AMP would tend to indicate a lack of assay specificity.

The results of Manganiello and Vaughan (1972), who reported values of 10 to 16 pmol cyclic AMP per mg protein in L929 cells, are within the range of values obtained in my experiments. Their cells were plated at a two-fold lower density than mine (0.9×10^6 cells per dish) using identical dishes, and incubated 3 to 4 days before cyclic AMP measurements were made. Assuming time taken to reach the logarithmic growth period is related to density of plating (Fig 18) their cells would possibly have been in the early growth period when assayed. Their measurements are higher than those of 6 to 9 pmol cyclic AMP per mg protein obtained in this thesis during the early growth period.

Otten et al (1971) quoted a value of 21 pmol cyclic AMP per mg protein for L929 cells in logarithmic growth. During logarithmic growth in the two series of experiments quoted above, intracellular cyclic AMP fluctuated from a minimum of 3.7 to a maximum of 9.2 pmol/mg protein. Such fluctuations in cell cyclic AMP during different stages of the logarithmic growth phase do not support the hypothesis that growth rates

of cultured cells are inversely proportional to intracellular concentrations of cyclic AMP, based on an apparently constant level of intracellular cyclic AMP measured "during logarithmic growth" (Heidrick and Ryan 1971; Otten et al, 1971; 1972; see also Fig 40)

Inter-laboratory variations in methodology and assay validity have tended to make quantitative comparisons difficult.

Qualitative Comparisons with Other Investigators

The elevated cyclic AMP at the lag period, 24 hours before logarithmic growth commenced, is qualitatively similar to results found with confluent cultures of non-transformed ("contact" inhibited) fibroblast lines (Froelich and Rachmeler, 1972; Anderson et al, 1973; Rudland et al, 1974 b) and with the report that cell cyclic AMP levels are highest at or near the beginning of the S phase (Chlapowski et al, 1975). On the other hand, the fact that cell cyclic AMP remained constant during the early growth period until 24 hours after the lag period had begun, is consistent with the reports which say that cyclic AMP does not rise on confluency in transformed cell lines (Otten et al, 1971; Sheppard, 1972a; Burstin et al, 1974). Such ambiguity of reported results amplifies the necessity in future experiments to monitor in detail the point in growth at which cell-cell contact is complete and confluency has occurred, eg by time-lapse cinematography (Castor, 1968).

However, qualitative comparisons with other investigators tend to be more meaningful. It has been convincingly documented (see Sheppard, 1972a, Ryan and Heidrick, 1974) that a rise in cell cyclic AMP tends to be associated with a decrease in growth rate in cultured cells, as occurred during the lag and

decline phases in my experiments.

Intracellular Cyclic AMP During Growth Inhibition by Ethanol

During the early logarithmic growth period in control cells, the extended lag phase or decreased growth rate of the ethanol treated cells was reflected by elevated intracellular cyclic AMP. Since at this point, control cultures had more mitotic cells than experimental cultures and cyclic AMP is reported to be lowest during the S and M phases of the cell cycle (Ryan and Heidrick 1974), this result is consistent with delayed entry into S phase in ethanol treated cells. Inhibition or suppression of growth in cultured cells resulting from treatment with agonists which increase intracellular cyclic AMP arrests cells in the G_1 phase of the cell cycle (Frank 1971; Rosengurt and Pardee 1972; Willingham et al 1972; Zimmermann and Raska 1972; Bombik and Burger 1973).

DNA, RNA AND PROTEIN

Lindsay (1969) reported that decline phase ("stationary") populations of L929 cells had a greatly reduced capacity to synthesise DNA in comparison to rapidly dividing cultures. My results can be interpreted as confirming Lindsay's findings, since intracellular DNA tended to fall during the growth decline period. In my experiments, as reported elsewhere (Swaffield and Foley 1960; Ward and Plagemann 1973), the cells had apparently synthesised DNA, RNA and protein over the period between trypsinisation and 24 hours after plating out. Nevertheless, entry into S phase subsequent to day 1 was suppressed until logarithmic growth commenced, when culture DNA increased.

In the first series of experiments, population increases of 34% and 26% respectively during the two day early growth period, in the presence of DNA increases (per culture) of 11% and 3% respectively, indicate that a minority of the population were passing directly from G_2 to G_1. Since L-cells are polyploid (Hsu and Klatt 1958), cells passing from G_2 to G_1 via mitosis, without concomitant DNA synthesis is a feasible event and may have been a means of increasing the population density to allow a reasonable survival probability (as discussed; page 76). Gelfant (1962) reported a resting "G_2 population" amounting to 16% of the cells in mouse epidermis. Such a condition is found in the myocardial cells of man (Sandritter and Scomazzoni 1964). The significant rises in RNA and protein per culture which occurred in the absence of rises in DNA are consistent with cells progressing from G_2 to G_1 but being prevented from entering the S phase.

The early growth period was not present in the second series of experiments, or in those of Lindsay (1969), where cells were plated at a higher cell density. This means that a larger proportion of the population were in G_2 when logarithmic growth commenced in the second series of experiments, implying that growth would have been less synchronous. Similarly, this would disguise any occurrence of a rise in culture RNA preceeding a rise in culture DNA, since cell division would have occurred in a biphasic manner.

Further similarities may be seen with respect to the lag phases in the two series of experiments and "contact" inhibited growth at confluency:

(i) decreased DNA synthesis (Schutz and Mora 1968; Pontén et al 1969; Griffiths 1971);

(ii) decreased RNA and protein synthesis (Levine et al 1965; Griffiths 1971).

(iii) A loss of protein from the cell has been reported (Kruse et al 1967)
as well as the elevated intracellular cyclic AMP. Contact inhibited cells are arrested in the G_1 phase of the cell cycle (Nilausen and Green 1965).

The above events also tended to occur when cells entered the decline phase of growth. A drop in cell protein preceeded the drop in cell DNA and RNA in the first series of experiments. In the second series of experiments these events occurred simultaneously. Since the magnitude of the terminal cell density has been reported to be related to the serum content of the medium (Castor 1971; Westermark 1971) the decline in

cell protein may be part of the endogenous mechanism which determines the timing of growth decline as described by Skehan (1976).

Between two and three days after commencement of the growth decline period, a drop in cell numbers (Fig 29) occurred simultaneously with a rise in cell DNA and RNA (Fig 33). It is likely that this took place as lost cells were replaced from the monolayer. The levels reached of cell DNA and RNA were still low in comparison to lag phase or logarithmically growing cells.

Macromolecular Events When "Resting" Systems are Stimulated to Divide

In various other systems where cells arrested in G_1 are stimulated to proliferate, eg

(a) liver regeneration after partial hepatectomy (Brues et al 1944; Ultmann et al 1953; Giudice and Novelli 1963; Giudice et al 1964).

(b) kidney cortex cells freshly explanted from the animal (Lieberman et al 1963a,b)

(c) confluent, "contact" inhibited cells after the addition of serum (Todaro et al 1965, 1967)

(d) rat hepatoma (Ward and Plagemann 1973) and mouse L-cells (Weissman et al 1960; Lindsay 1969) subcultured from decline phase populations, the authors above reported or suggested that initiation of DNA synthesis was preceeded by the synthesis of specific RNA and protein. On this basis, the sequence of events in initiation of DNA synthesis could be compared to enzyme induction in bacteria (Kepes 1963) and higher animals (Pardee and Wilson 1963) or to the mechanism of action of certain hormones (Hamilton 1964) and could be

summarised as follows: An environmental stimulus, eg a critical concentration of a nutrient or modification in the physical state of a cellular molecule may allow the synthesis of a template RNA, resulting in the enzymes involved in the replication of the DNA.

Thus elevated rates of RNA synthesis are required for subsequent DNA synthesis during 12 to 22 hours after establishment of rabbit kidney cortex cells in culture. Low levels of Actinomycin-D which abolish this rise in rate of RNA synthesis while not preventing the normal rates of RNA turnover have been shown to prevent the subsequent rise in DNA. The induction of a new species of messenger RNA seems to be the Actinomycin-D sensitive process (Fujioka et al 1963).

THE INTERVENTION OF ETHANOL IN STIMULATION OF CELL DIVISION

Ethanol addition resulted in an alteration in the control of cell division. A cell that has synthesised DNA is one which has made the decision to divide (Baserga 1965). An alteration in the control of cell division (Swann 1957, 1958) then becomes some alteration in the initiation of DNA synthesis. Factors that may intervene in the temporal regulation of DNA synthesis are as follows:

(i) the regulation of the synthesis of RNA and proteins

(ii) the availability of DNA percursors

(iii) the physical state of the DNA molecule

(i) Possible Effects of Ethanol in Influencing the Synthesis of RNA and Proteins

Ethanol treated cells tended to have lower DNA per culture during the lag phase of growth in control cells, when cell populations in control and experimental dishes were identical. This DNA was used as the template for synthesis of RNA during the latter half of the lag phase. Ethanol treatment tended to decrease, but did not prevent the rise in RNA which preceeded the rise in DNA before commencement of logarithmic growth. This perhaps suggests that ethanol did not interfere qualitatively with transcription. During the first 24 hours of logarithmic growth in the first series of experiments, there was some rise in culture protein at the suppression of cell division in ethanol treated cells. Interpretation of results at this point in growth is difficult due to differing growth states of control and experimental cells. However, DNA per culture in ethanol treated cells was the same or lower than lag phase

control cells, whereas protein per culture was significantly higher at this point than in lag phase control cells. This implies that ethanol did not interfere with general protein synthesis from RNA in G_1. In the second series of experiments, where the rise in RNA was more dependent upon de novo synthesised DNA, addition of ethanol simultaneously suppressed rises in culture DNA, RNA and protein during the first 24 hours after its addition. The results amplify the suggestion that suppression of growth by ethanol was a result of delayed entry into S phase in treated cells.

Furthermore, experimental cells, being poor in DNA, were also poor in RNA. Protein synthesis was not so markedly affected. However it is known that protein synthesis can occur independently of DNA synthesis (Rueckert and Mueller 1960; Churchill and Studzinski 1970). However to completely exclude ethanol interference with general RNA and protein synthesis in G_1 the experiments carried out would have to take account of their rates of synthesis per unit DNA in the presence and absence of ethanol, eg ^{3}H-uridine uptake and ^{3}H-leucine uptake.

Small (but significant; Figs 19and 30) increases in cell population in the absence of significant rises in culture DNA (Figs 26 and 35) implies that during these periods, cells in G_2 were not prevented from dividing and entering G_1. Thus when ethanol treated cells divided, they did so with a greater degree of synchrony than control. An experiment could be carried out to further justify this conclusion by estimating cell population over consecutive time intervals which are

small in comparison to the length of the cell cycle. Data from counts alone gives degree of synchrony (Engelberg 1961).

The Possibility of an Induction Mechanism in Ethanol Treated Cells

The inhibition of growth was a temporary effect: ethanol treated cells eventually proliferated. Examination of the results indicates that ethanol did not appear to interfere qualitatively with RNA and protein synthesis. However, the evidence is consistent with induction of enzymes for replication of DNA as an event occurring before logarithmic growth as has been described for the model systems above. It is possible therefore that during the extended or initiated lag phase in experimental cells, they may have been occupied in the induction of a specific mechanism to compensate for nutritional and/or physical changes in the cell brought about by the presence of the ethanol molecule. Thus, red blood cells of alcoholics have been shown to increase the activity of the sodium pump to compensate for increased intracellular $[Na^+]$ brought about by alcohol (Lindsay 1974a). The increase is thought to be a result of de novo synthesis of protein (Lindsay 1974b). Other investigators have provided evidence for the involvement of the nucleus in alterations in sodium pump density in response to changes in intracellular $[Na^+]$ (Boardman et al 1975). Raising extracellular $[K^+]$ has been shown to interfere with amino acid transport into L929 cells (Kuchler 1967). The nucleus has been shown to be important for the maintenance and modification of amino acid transport

in HeLa cells (Hume et al 1975). Bacteria removed from a glucose-containing medium and placed in a lactose-containing medium enter a lag phase in growth during which they synthesise enzymes for metabolism of the lactose. Subsequently, logarithmic growth can occur (Mandelstam and McQuillen 1973).

By analogy with the evidence above, such a mechanism in ethanol-treated cells would require transcription of previously "masked" genes to produce a new species of RNA with subsequent synthesis of an enzyme to counteract the metabolic changes produced by the ethanol molecule. Logarithmic growth would be delayed until completion of this compensatory mechanism.

(ii) Possible Effects of Ethanol on Availability of DNA Precursors

It is possible that ethanol may have prevented the increased culture DNA which occurred at logarithmic growth in control cells by affecting the uptake into the cell of nucleotides by a perturbation of the membrane structure. As discussed in the Introduction (page 10) non-specific depolarisation of the cell membrane by ethanol has been exhibited in many situations *in vivo*, even in those where depolarisation does not normally occur. As stated above, raising extracellular $[K^+]$ has been shown to interfere with amino acid transport into L929 cells (Kuchler 1967). An experiment involving nucleotide transport eg measurement of ^{3}H-thymidine incorporation into total cellular material would be a means of further investigation.

(iii) Possible Effects of Ethanol in Affecting the Physical State of the DNA Molecule

Ethanol is known to be capable of entering the cell by simple diffusion across the cell membrane. Although I could see no evidence of metabolic degradation of ethanol, it is feasible that a small amount entering the cell could affect

the physical state of the nucleus. Any perturbation of the membrane structure by ethanol could feasibly affect the physical state of the DNA molecule without ethanol having to enter the cell. Ethanol has been shown to produce quantitative changes in membrane lipids (Miceli and Ferrell 1973). With reference to the ability of ethanol to influence ion transport, raising extracellular $[K^+]$ has been shown to inhibit DNA synthesis within minutes in baby hamster kidney cells, an effect which preceeds any significant change in intracellular $[K^+]$. (Orr et al 1972).

CYCLIC AMP IN RELATION TO DNA, RNA AND PROTEIN

According to the present hypothesis in the literature of the role of cyclic AMP in cell growth, the peak in intracellular cyclic AMP which occurred 24 hours before logarithmic growth commenced could be related to:

(i) Initiation of DNA replication. A transient increase in cyclic AMP has been observed to occur before DNA synthesis in thymic lymphocytes stimulated to divide using various prostaglandins, which raise intracellular cyclic AMP (MacManus et al, 1975). Parallel changes in cyclic AMP levels and DNA synthesis have been achieved in vivo by the infusion into partially hepatectomised rats of an empirically devised hormone mixture containing triiodothyronine (T_3) and glucagon (MacManus et al, 1975). These authors reported a peak in cell cyclic AMP 12 hours after partial hepatectomy, which if prevented resulted in suppression of the rise in DNA at 18 hours.

(ii) Events resulting in the synthesis of a specific messenger RNA, where elevated cyclic AMP and the presence of the lag phase may be related to induction of specific enzymes necessary for uptake and utilisation by the cell of specific nutrients. By analogy with enzyme induction in bacteria, where cyclic AMP is required for the transcription process itself (Mandelstam and McQuillan, 1973) the nutrients would be "inducers" and the cyclic AMP a "mediator" of the induction mechanism. The evidence to suggest such an occurrence in mammalian cells is as follows:-

(a) Addition of serum to cultured cells results in decreased cell cyclic AMP. For certain cell types, the promotion of protein synthesis and cell proliferation by serum factors is definately a function of serum concentration (Amos, 1967; Temin, 1967; Todaro et al, 1967). Elucidation of the factors ("inducers") which exert regulatory effects on cells in vitro has been attempted. Highly fractionated and partially characterised serum conponents have been found in part to replace the stimulatory activity of serum for cell proliferation (Todaro et al 1965; Holmes 1967; Puck et al 1968).

(b) Insulin has also been found to exert considerable such activity (Gey and Thalimer 1924; Schwartz and Amos 1968). Insulin lowers intracellular cyclic AMP in vitro (Sheppard 1972a, b; Bombik and Burger 1973; Scher et al 1974), whether it exerts such an effect in vivo is controversial (Pastan and Perlman 1971; Wicks 1974). In vivo, insulin stimulates sugar (Levine et al 1949, 1950) and amino acid (Wool et al 1965) transport, and increases protein synthesis, the latter being an effect believed to be brought about by the translation of synthesised RNA (Wool et al 1968).

(c) As stated in the Introduction, a prominent action of cyclic AMP dependent protein kinases is their ability to phosphorylate histones (Langan 1969). The proposed role of histones, the basic protein associated with DNA, is to regulate gene transcription, and the phosphorylation of histones has been proposed to uncover gene sequences allowing them to be transcribed (see Pastan and Perlman 1971). In liver cyclic AMP is involved in the induction

of various enzymes, including tryosine transaminase, phosphenol pyruvate carboxykinase and serine dehydratese. (Wicks et al 1969).

In accord with (a), (b) and (c) above, the rise in cyclic AMP would result in transcription of RNA to be "translated" by serum factors. A subsequent fall in cyclic AMP would signify completion of the induction mechanism.

The requirement for an enzyme induction mechanism may not have been present until the lag phase, where, for example, confluency of the cell sheet may have hindered diffusion of nutrients across the cell membranes. The addition at confluency of fresh medium containing at least 10% serum has been reported to result in cell division in many cell lines which normally show density dependent inhibition of growth (Kruse et al 1969) and are presumably arrested in G_1 (Nilausen and Green 1965).

(iii) The occurrance of low protein per cell in comparison to the phases of more rapid growth (early and logarithmic). Cyclic AMP reached a maximum during the two days after commencement of the growth decline phase in the second series of experiments (days 5 to 7 of Fig 31) when cell protein reached a minimum. Elevations in intracellular cyclic AMP and decreases in intracellular protein both appear to occur in lag phase and decline phase growth. Cell cyclic AMP was related to cell protein by a negative log linear relationship in my experiments (Fig 39). In this respect, an elevation in cyclic AMP could be a response to, as well as a possible mediator of, changes in metabolism. The elevated cyclic AMP in cells whose growth rate was low (lag and decline phase cells) may be a reflection more of the availability of crucial nutrients to the cell rather

than their absence in the medium (Sheppard 1972a; Seifert and Paul 1972) since the medium was changed daily in my experiments.

Since adenyl cyclase is a membrane bound enzyme (Sutherland et al 1962) it is feasible, as suggested by Bannai and Sheppard (1974) that changes in cyclic AMP levels are a reflection of an altered nutritional environment eg a crucial degree of cell-cell contact at the lag phase; a crucial degree of cell overlap at the decline phase. Bannai and Sheppard (1974) reported cell contact in transformed (3T6) cells at 0.370 mg protein/10^6 cells at a cell density of 4.4×10^4 cells/cm^2. In my model, 24 hours after logarithmic growth commenced, mean cell densities in control cells were 4.8×10^4 and 4.3×10^4 cells/cm^2 respectively, with mean protein contents of 0.370 and 0.437 mg/10^6 cells respectively in the two groups of experiments. The 7% increase in cell numbers between days 4 and 5 in the first series of experiments, parallelled by a 122% increase in cell cyclic AMP, may have been sufficient to bring the cells from a precontact to a contact state. Since many cell membrane parameters change as a function of normal cell contact (eg nutrient transport, membrane enzyme activity; see Introduction) the decrease in cyclic AMP which occurred when the cells began to grow logarithmically may not have been the primary event which allowed the increases in cell protein, RNA and DNA, and cell numbers observed. However, since agents which increase intracellular cyclic AMP have been shown to suppress growth, while agents which decrease intracellular cyclic AMP have been shown to stimulate growth (see Introduction) it seems likely that an increase in cell cyclic AMP is a critical event which responds to the availability to the cell of crucial nutrients.

In this respect the decrease in cell cyclic AMP produced by insulin (above) could be interpreted as a result of increased glucose and amino acid transport, or vice versa. Similarly glucagon acts purely at the cytoplasmic level, increasing cyclic AMP and promoting glycogen breakdown (Fig 4). This action could in turn increase glucose transport. The decreased cell cyclic AMP of actively dividing cells in comparison to cells of the slow growth periods (lag and decline) may be due for example to increased motility, aiding diffusion (Castor 1970, 1971). Since in many situations *in vivo* in which cells show a high mitotic activity, they are moving in relation to each other (Carter 1968).

Cyclic AMP in Relation to the Nutritional State of the Cell

Any relationship of cyclic AMP to the nutritional state of the cell would not exclude it from mediating or potentially mediating any subsequent change by the mechanisms proposed by (i) to (iii) above, or by some other mechanism. The above evidence implies that inhibition of growth can be avoided if the cell is able to take up an adequate supply of nutrients in a crowded environment.

(i) Cyclic AMP in Ethanol Treated Cells in Relation to Initiation of DNA Synthesis

Increased intracellular cyclic AMP was concurrent with a suppressed rise in DNA content per culture in ethanol treated cells during the first 24 hours of logarithmic growth in control cells. It is possible that the two events may be directly related. However, the decrease in cell cyclic AMP may not have been the primary event which resulted in DNA synthesis. My results imply that ethanol prevented entry into S phase without affecting the general rises in RNA and protein which preceeded

it. In transformed cells whose growth in inhibited by dibutyryl cyclic AMP or agonists which maintain high intracellular cyclic AMP, the suppression of DNA synthesis has been reported to be parallelled by an increase in RNA and protein synthesis (Lim and Mitsunobu 1972; Van Wijk et al 1972; Curtis et al 1973; Korinek et al 1973; Kram et al 1973). Since such cells are inhibited in G_1, this could be the consequence of continued synthesis of RNA and protein while entry into S phase is prevented.

(ii) Cyclic AMP in Ethanol Treated Cells in Relation to Synthesis of a Specific Messenger RNA

Ethanol did not appear to interfere with general transcription which preceeded the first round of DNA replication. However, assuming an enzyme induction mechanism in control cells during the lag phase, with the resultant synthesis of a new species of messenger RNA, ethanol may have prevented the synthesis of this new RNA. Alternatively, ethanol may have required the synthesis of a messenger RNA specific to ethanol treated cells to compensate for changes in metabolism produced by ethanol (cf evidence for the induction of $(Na^+ + K^+)$-stimulated ATPase to compensate for increased intracellular $[Na^+]$ produced by ethanol in HeLa cells; Lindsay 1974b). Assuming the direct participation of cyclic AMP in the transcription process, as proposed by its ability to phosphorylate histones, the elevated cyclic AMP during the lag in ethanol treated cells, when control cells were growing logarithmically, may account for such a mechanism. Adding low amounts of Achnomycin D (cf Fujioka et al 1963) would give an indication of experimental cells inducing a new species of messenger RNA while control cells were not. Hybridisation studies (Church and McCarthy 1967a,b) could be used to detect a species of messenger RNA in ethanol treated cells, not present in control cells.

(iii) Cyclic AMP in Ethanol Treated Cells in Relation to Cell Protein

In the first series of experiments, ethanol blunted the rise in cyclic AMP during the lag phase, when cell protein tended to be higher in ethanol treated cells; during growth decline in both series of experiments protein content tended to be higher in experimental cells while cyclic AMP content tended to be lower. In fact most fluctuations in cell cyclic AMP during growth in ethanol treated cells in comparison to control could be anticipated by the log linear relationship of intracellular cyclic AMP to intracellular protein (Fig 39). Furthermore during the first 24 hours of the growth decline period the fall in cell protein, together with a rise in cell cyclic AMP were the only consistent variations of the parameters measured to occur in control and experimental dishes in the two series of experiments. Together with the evidence indicating a decrease in cell protein as the primary event of those measured to occur before growth decline, this implies a relationship between cell protein, cell cyclic AMP and growth rate.

In the short term experiments carried out in serum-free medium, serum depletion raised intracellular cyclic AMP in control cells and ethanol addition apparently achieved this effect more rapidly. The elevation in cyclic AMP in these experiments cannot be related to an induction mechanism per se, since there were no "inducers" present in the medium, and the same effect occurred at various cell densities (corresponding to early growth, lag and logarithmic growth). The response is probably comparable to the stress response postulated by Sheppard (1972b).

Cyclic AMP in Ethanol Treated Cells in Relation to the Nutritional State of the Cell

Replication of DNA was delayed after ethanol treatment in comparison to control; experimental cells were affected at some point in the G_1 phase of the cell cycle. If ethanol suppressed cell division by affecting:

(i) the synthesis of specific RNA and proteins;

(ii) the availability of DNA precursors; or

(iii) the physical state of the DNA molecule,

then the elevated cyclic AMP which resulted in ethanol treated cells could be related to an effect on the nutrition of the cell.

Assuming a "response" of cyclic AMP, as proposed by its postulated role as a regulator of cell metabolism, and by analogy with

(a) an increase in cell cyclic AMP being the signal involved in nutritional deficiency in mammalian cells (Sheppard 1972b) as well as in bacteria (Mandelstam and McQuillen 1973);

(b) the in vivo connection of cyclic AMP with hormones of energy metabolism and stress (Table I);

the elevation in cyclic AMP in experimental cells may have been related to mediating a compensatory mechanism which released the inhibition on growth. Whether the subsequent growth of ethanol treated cells was a result of:

(i) release of inhibition on DNA synthesis;

(ii) an increase in synthesis of a specific messenger RNA;

(iii) increased availability of general and/or specific nutrients to the cell;

(iv) a combination of (i) to (iii) above,

the evidence strongly suggests than an enzyme induction mechanism was involved. This reasoning has the advantage that it provides a unitary explanation for events in control and experimental cells and gives an analagous type of role to cyclic AMP to that found in bacteria. Thus during the lag phases in growth, the cells may have been occupied in a specific induction mechanism to compensate for changes in metabolism brought about by, eg cell-cell contact. The extended lag phase in ethanol treated cells may have been due to some kind of inhibition of this mechanism by ethanol. However the finding that ethanol treated cells "recovered" within 24 hours to grow at least as rapidly as control cells implies that experimental cells were themselves occupied in an induction mechanism to compensate for changes in metabolism brought about by the presence of the ethanol molecule. The results suggest that elevated cyclic AMP may have been involved in the mechanism.

POSSIBLE RELATIONSHIPS OF THE RESULTS TO IN VIVO EFFECTS OF ALCOHOL

Ethanol interfered temporarily with the growth of the cells, without affecting their viability and apparently without being metabolised by the cells. In view of the known ability of ethanol to penetrate cell membranes (Kalant 1971) it is likely that some kind of membrane change occurred. Ethanol is potentially capable of reaching the cell membrane of any cell of the body (Williams 1975).

The results in this thesis imply that where an increased cell cyclic AMP occurred in experimental cells, it was related to an effect on the nutrition of the cell. That the response was non-specific is in general agreement with the changes found by the authors in Tables II and III , with the possible exception of the results of Gorman and Bitensky (1970).

Regarding the postulated role of cylic AMP as the "second messenger" in the actions of the hormones illustrated in Table I , any non-specific changes in cyclic AMP occurring in the cells of the body could feasibly affect systems as diverse as impulse induction, neurotransmitter and catecholamine release and actions, water permeability. The evidence that activation of adenyl cyclase in vivo often requires a certain ionic environment (Brodie et al 1966: Robison et al 1971) is of additional significance if any change produced by ethanol affected the ionic potential of the cell membrane. For example, the interference of ethanol in the nutritional state of the cell could be extrapolated to the reports of decreased uptake of neurotransmitters into synaptosomes (Roach et al 1973) amino acids into brain in vivo (Choy et al 1972; Freud 1972), reduced intermediary metabolism in the brain (Veloso et al 1972) as a result of ethanol

treatment, all of which may be simply reflections of decreased neuronal activity as a result of cell membrane perturbation.

Tolerance

Despite continued addition of ethanol-containing growth medium, experimental cells grew logarithmically approximately one day later than control cells. A compensatory mechanism occurring over this one-day period allowed ethanol treated cells to grow logarithmically. That this mechanism involved enzyme induction is a feasible possibility, consistent with the reports that physiological tolerance to alcohol may involve increased synthesis of $(Na^+ + K^+)$-ATPase in brain (Israel et al 1970) possibly contributing to adrenergic supersensitivity (French et al 1975); in liver, contributing to the "hypermetabolic state" (Berstein et al 1974; Israel et al 1975) and other tissues (Lindsay 1974a,b). By analogy with similarities between thyrotoxicosis and chronic ethanol treatment and with the evidence that thyroid hormones increase adenyl cyclase synthesis (Brodie et al 1966), the adrenergic supersensitivity produced by ethanol may be a result of increased adenyl cyclase activity (Israel et al 1972; Kuriyama and Israel 1973).

The advantage of using a whole cell system, free from hormonal perturbations whereby changes in cyclic AMP were reflected by changes in growth rate, allowed monitoring of cyclic AMP changes without inadvertently affecting the physical state of the adenyl cyclase molecule. By analogy with bacteria (Mandelstam and McQuillen 1973) cyclic AMP may have played a role in the compensatory mechanism whereby the experimental cells could grow. The results do not dispute the possibility discussed in the Introduction, that the same type of molecular action, involving the

cell membrane, may be included in all the effects of ethanol on the body.

Possibilities for Further Investigation

As well as the suggestions cited in the text, possible modes of further investigation could include the molecular mechanism of ethanol interference. Thus radioactively-labelled ethanol could be usefully employed to investigate the degree of penetration of ethanol into the cell membrane and any entry into the cell. Further studies could be carried out to examine the growth and cyclic AMP changes in experimental cells in relation to ionic perturbations and hormonal responses. Comparisons with results observed using primary cell systems (eg kidney cortex cells, connective tissue) isolated and subcultured from previously intoxicated experimental animals would help in relating *in vivo* phenomena.

A knowledge of the quantitative levels of cyclic AMP during the various phases of the cell cycle would be of value in investigating the direct site of action of ethanol in the cell cycle. Experiments involving isolation, characterisation and comparison of the species of messenger RNA formed by control and experimental cells would investigate the presence of an enzyme induction mechanism specific to ethanol treated cells. Similarly, the possibility of de novo protein synthesis in experimental cells as a result of an enzyme induction mechanism could be regarded as a feasible hypothesis for further investigations. The specificity of the cyclic AMP response in experimental cells could be further investigated by monitoring adenyl cyclase and phosphodiesterase activities in whole cell preparations.

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