THE EFFECTS OF VANADATE IONS ON CULTURED MAMMALIAN CELLS

by

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SUMMARY

The effects of vanadate ions were investigated on cell growth, migration, spreading, DNA and protein synthesis and protein secretion of mammalian cells in culture. It was found that vanadate inhibits the spreading of cells on fibronectin (at concentrations of $1x10^{-4}$ - $1x10^{-5}$M) and that inhibition can be reversed by washing or the addition of arterenol to reduce and chelate the ion. Vanadate also causes the rounding up of pre-spread cells. Transformed cells (Py and ASV cells)were more sensitive than their normal counterparts (C13 cells).

Incubation of the cells for more than 12 hours with vanadate at concentrations of 10^{-5}M and above, inhibited growth and reduced plating efficiency. DNA and protein synthesis in intact cells are not sensitive in the short term even at concentrations reported to be inhibitory to certain isolated component enzymes of these pathways in vitro.

It is therefore proposed that either the entry of vanadate is poor or, more likely, vanadate enters freely but is chemically altered (?reduced and complexed) so that the internal concentrations probably do not approach those required to inhibit many enzymes in vitro.

Vanadate could be affecting specific, highly sensitive targets to bring about its effects. However of the enzymes known to be inhibited by vanadate evidence is presented that neither the Na/K ATPase, adenylate cyclase or dynein-like ATPase are likely to be involved in the inhibition of spreading.

An alternative possibility is considered that the inhibition could be mediated by an effect on protein phosphorylation.

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CONTENTS

ABBREVIATIONS

ASV cells	Avian Sarcoma virus, Schmitt Ruppin variant, transformed BHK cells
BHK-21 cells	Baby Hamster Kidney cells
C13 cells	Clone 13 BHK-21 cells
cAMP	Cyclic Adensoine 5'Monophosphate
CS	Calf Serum
dbCAMP	dibutyryl Cyclic Adenosine 5'Monophosphate
DNA	Deoxyribonucleic acid
DNAase 1	Deoxyribonuclease 1
DMSO	Dimethyl Sulphoxide
DTPA	Ca-Na_3-Diethylene Triamine Pentaacetic Acid
ECT	Bicarbonate buffered Eagles medium
EDTA	Ethylene Diamine Tetra-Acetic Acid
EGF	Epidermal growth factor
EM	Electron Microscopy
ER	Endoplasmic reticulum
ESR	Electron spin resonance
FN	Fibronectin
GDP	Guanosine diphosphate
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine triphosphate
HECT	Hepes buffered Eagles medium
HH	Hanks Hepes
HMG CoA Reductase	3-Hydroxy-3 methyl glutaryl-CoA reductase
HS	Hepes Saline
IBMX	Isobutyryl-1-Methyl Xanthine
IF	Intermediate Filament
Kd	Kilo dalton
mf	Microfilament

Mr	Apparent molecular weight
mt	Microtubule
NADH	Nicotinamide Adenine Dinucleotide Hydride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydride
PAH	Para Amino Hippurate
PBSa	Phosphate Buffered Saline
PDGF	Platelet derived growth factor
PMSF	Phenylmethyl suphonyl fluoride
ppb	Parts per billion
ppm	Parts per million
PPO	2,5 Diphenyloxazole
PY cells	Polyoma transformed BHK cells
RNA	Ribonucleic acid
RNAase	Ribonuclease
RPM	Revolutions per minute
RSV	Rous sarcoma virus
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
TAME	p-Tosyl-l-arginine methyl ester-HCL
TCA	Trichloro acetic acid
TEM	Transmission Electron Microscopy
TPB	Tryptose Phosphate Broth
V	Versene

INTRODUCTION

Vanadate ions have been shown to inhibit the aggregation of fibroblasts in suspension (Edwards 1981) to the same extent as colchicine and other microtubule poisons (Waddell et al 1974). Vanadate also alters the intermediate filament distribution of cultured fibroblasts, forming the characteristic perinuclear coils of intermediate filaments in a mannner similar to that of colchicine. The vanadate apparently does not affect the distribution of the microtubules (Wang and Choppin 1981). Thus it is of interest to investigate the effects of vanadate on cellular functions dependent on cytoskeletal systems.

To help understand these phenomena I have investigated a variety of effects of vanadate on intact cells. This introduction consists in the main of a review of known biological effects of vanadate.

VANADIUM

Biological Interest In Vanadium.

Until recently vanadium was viewed by biologists as a rather exotic element, although it was found in relatively high concentrations in some species of ascidians (Carlson 1975), and Amanita muscaria (Schwartz and Milne 1971). Vanadium was recognised as a trace element in many organisms (Underwood 1977),

but it had no known biological function. Even now no vanadium-containing enzymes have been described and the effects of exogenous vanadium on metabolism are not understood. However in 1977 Cantley et al identified a potent inhibitor of the Na/K ATPase of erythrocytes that was present in Sigma grade ATP, from equine muscle, as an impurity. The inhibitor was vanadium (V) i.e. vanadate. They suggested that vanadate may act in vivo to regulate the Na/K ATPase (Cantley et al 1977). A considerable effort has since ensued to try to elucidate the effects of vanadate on biological systems (e.g. Carpenter 1981; Smith 1983 and see below) and it is this that lies at the basis of this work.

Vanadate has since been excluded from playing a physiological role in regulation of Na/K ATPase since it is thought that the vanadium exists in the cell as the vanadium 4+ vanadyl form (Macara et al 1980) (see below) but nevertheless has proven to be a useful tool for investigating a whole range of phenomena (Kustin and Macara 1982) as I hope to illustrate below. It must be noted, however, that there are a few reports in the literature of vanadium 4+ inhibiting enzymes in vitro (see Table 2 and DeKoch et al 1974; Menon et al 1980.)

Physical Characteristics

The element vanadium is named after the Scandinavian Goddess Vanadis, apparently a reference to its multi-coloured compounds. It was first discovered by Sefstrom in 1830 and isolated by Roscoe in 1869 (Strasia 1971). It forms about 0.01% of the earth's crust and is found in several minerals, including Vanadinite and Patronite (Strasia 1971).

Vanadium belongs to the first transition series, a metal in Group V of the periodic table. The natural element is a mixture of 2 isotopes ^{50}V (0.24%) and ^{51}V (99.76%). ^{51}V is slightly radioactive with $t1/2=1x10^{15}$ years. Six isotopes are recognised, numbered 47-52. Biologically useful isotopes that are commercially available are ^{48}V and ^{49}V (Falkner-Hudson 1964).

Basic Chemistry

Oxidation States

Vanadium is found in +2 (vanadous), +3 (vanadic), +4 and +5 oxidation states. In this thesis the ions referred to in the +4 oxidation state are the vanadyl VO^{2+}, and in the +5 oxidation state the vanadate $H_2VO_4^-$ ions.

Vanadium +2

In this valency the vanadous ion behaves as a divalent cation, resembling Fe^{3+} or Cr^{2+}, but is more readily oxidised than chromium, and decomposes rapidly in neutral solution in water, although decomposition can be retarded in the presence of concentrated acid. The V^{2+} ion is violet.

Vanadium +3

Vanadium 3+ is unstable at physiological pH and also in the presence of dissolved oxygen. It is stable at, and below, pH 2. It is a reducing agent and on warming can reduce copper sulphate solution to the metal. The ion is green/blue and behaves as a

Table 1

Vanadium : Oxidation states and potentials

V5	vanadates vanadyl	colourless
V4	Vanadites vanadyl Hypovanadites	blue/variable
V3	Vanadites	green/blue
V2	vanadous vanadyl	violet

Where redox potentials are :-

$V^{2+} + 2e^- \rightleftharpoons V$	-1.175
$V^{3+} + e^- \rightleftharpoons V^{2+}$	-0.255
$V^{5+} + e^- \rightleftharpoons V^{4+}$	-0.74
$VO^{+2} + 2H^+ + e^- \rightleftharpoons V^{3+} + H_2O$	+0.337
$VO_2^+ + 2H^+ + e^- \rightleftharpoons VO^{2+} + H_2O$	+0.991
$V(OH)_4 + 2H^+ + e^- \rightleftharpoons VO^{2+} + 3H_2O$	+1.00
$V(OH)_4 + 4H^+ + 5e^- \rightleftharpoons V + 4H_2O$	-0.254

trivalent metal - similar to Fe^{3+} or Cr^{3+}. Unlike those of the +4 or +5 oxidation states the complexes formed by +3 ion are never vanadyl compounds. Nearly all the complexes are anionic and the co-ordination number is almost always 6.

Vanadium +4

Vanadium 4+ is also stable in acid pH, at and below pH3. Vanadites have the form $M_2V_4O_9$, and are few and unstable. Salts formed with acids, e.g. $VOSO_4$, are vanadyl compounds and are less readily oxidised than the vanadites. The VO^{2+} ion (vanadyl ion) is blue.

Vanadium 4+ has a paramagnetic, unpaired electron, and so can be detected by electron spin resonance (ESR) spectroscopy. On freezing, however, or when the ion binds to a macromolecule the signal changes dramatically. It is thus sensitive to the chemical environment and has therefore proved to be a useful probe of metal binding sites of several proteins. As ESR parameters are known for a wide variety of VO^{2+} complexes, ligands chelating VO^{2+} within proteins can be identified. In all cases so far studied imidazole and carboxyl groups are the ligands involved in VO^{2+} binding (DeKoch et al 1974; Kustin and Macara 1982). The disadvantage of such studies is the susceptibility of VO^{2+} to hydrolysis. Above pH 3 $VO(OH)^{+}$ begins to appear and the dimer then forms $(VOOH)_2^{2+}$. Above pH 4-5 a precipitate of $VO(OH)_3^{-}$ appears. At highly alkaline pH $VO(OH)_2$ forms, but little is known of the species occurring at intermediate pH (Macara 1980).

Vanadium +5

Vanadium 5+ compounds are usually colourless. The pentoxide, V_2O_5, dissolves in strong acids to form vanadyl salts of the type $[VO]X_3$. It forms few compounds, mainly with halides. At acid pH the dioxovanadium cation, VO_2^+, is found, but at physiological pH and above, the anionic vanadates $H_2VO_4^-$, appear. These tend to aggregate and form trimers ($V_3O_9^{3-}$)and tetramers($V_4O_{12}^{4-}$), at concentrations of $1x10^{-3}$M and above (Macara 1980) At lower pH the orange/yellow decavandate predominates. The dissociation rates of these polymers are slow (hours). At acid pH vanadium 5+ is a powerful oxidant and at pH 7 will oxidise aldehydes, catechols, olefins and sulphhydryls.

The orthovanadate ion, (predominantly $H_2VO_4^-$ at neutral pH) exists as a tetrahedral structure in solution, with a V-O bond length of approximately 1.66Å (Clark 1973). This shows great similarity to phosphate, which exists as a tetrahedral structure with a bond length of approximately 1.55Å. It has been suggested, in view of this, that vanadate may act as a phosphate analogue and bind to enzymes which it affects at sites that phosphate would normally occupy (Van Etten et al 1975; Lopez et al 1976; Kustin and Macara 1982). Vanadate ions have been shown to be potent competitive inhibitors of alkaline phosphatase catalysed hydrolysis of p-nitrophenyl phosphate in E.coli, the vanadate ions binding at the phosphate binding site (Lopez et al 1976), and to inhibit acid phosphatase hydrolysis of a variety of alkyl and aryl phosphate esters (Van Etten et al 1974). For a more detailed discussion of the chemistry see Sidgwick 1950; Clark 1968).

Vanadium As An Essential Trace Metal

Many characteristics common to essential nutrients are possessed by vanadium (Mertz 1970; Hopkins and Mohr 1974; Underwood 1977). Namely ;

Low molecular weight
Catalytic ability
Atomic structure
Transition metal with the ability to form chelates with biologically active compounds.
Ubiquitous distribution and has been generally available to plants and animals during their evolution.
Shown to be present in all plants and animals examined.
Low toxicity to most living organisms, especially mammals, when given orally.
Some sort of homeostatic mechanism is implied as in mammals, where serum levels are generally controlled, and there is a lack of accumulation and rapid excretion.
Diets deficient in vanadium induce characteristic symptoms in organisms.

An essential requirement for vanadium has been clearly demonstrated in Aspergillus niger (Bertrand 1942), Chlorella (Warberg et al 1955; Meisch and Beilig 1975) and Scenedesmus obliquus, where it is thought to be involved with photosynthesis (Arnon and Wessel 1953; Meisch and Beilig 1975). The thermophilic yeast Candida slooffii has shown a need for vanadium when grown at high temperatures (Roitman et al 1969). The trace element requirement seems widespread in algae, but has not been

shown in higher plants. Vanadium can substitute for molybdenum in some species of Azotobacter grown in molybdenum starved conditions. This is about 50% effective at enabling nitrogen fixation to proceed (Benemann et al 1972). In animals, characteristic deficiency symptoms include reduced wing and tail feather growth in chicks (Hopkins and Mohr 1973). Reduced body growth has been reported in rats fed low vanadium diets (Schwartz and Milne 1971; Strasia 1971). Reproduction has been shown to exhibit a marked impairment over several generations in rats fed on low vanadium diets (Hopkins and Mohr 1974). Fewer pups were born, compared to control vanadium-supplemented animals, and there was a much higher pup mortality at 21 days, than in controls.

Erythrocytes and iron levels were also affected as rats fed on a low vanadium diet had an increased packed cell volume of blood in comparison to control vanadium-supplemented animals. The blood and bone iron were significantly increased (Strasia 1971). Chicks fed on low vanadium diet also had increased packed cell volume (Nielsen and Ollerich 1973).

In vanadium-deficient chick tibiae the bone stucture is altered (Nielsen and Ollerich 1973), the epiphyseal plate is increased in size and there is a decrease in primary spongiosae. Lipid metabolism is also altered in animals deprived of vanadium. In chicks, serum levels of cholesterol were lowered initially (at 4 weeks) but after 7 weeks were significantly higher than in controls (Hopkins and Mohr 1973). Nielsen and Ollerich (1973) observed a similar phenomenon, with the serum cholesterol increase occuring after 4 weeks on the deficiency diet. Plasma triglyceride levels were doubled in vanadium-deficient animals.

Injection of radiovanadium shows that vanadium localises in areas of mineralisation, teeth and bones, and also in spleen, kidney and liver (Hansen et al 1982). Although the evidence suggests that vanadium is an essential element, its distribution and role within the body have not yet been elucidated.

Only 100 ppb dietary vanadium is required for normal growth in rats and 250 ppb provides optimal growth in cultured human fibroblasts. It has been proposed that most of the intracellular vanadium is present in the +4 oxidation state, complexed to small molecules such as ATP or glutathione or metalloproteins (Macara et al 1980; Degani et al 1981). If this were the case the suggestion of Cantley et al (1978a) that vanadate acts as a regulator of phosphohydrolases would seem unlikely. It is unlikely, also, to act as a transition metal cofactor in the form of VO^{2+} as this vanadyl ion can replace, and presumably be replaced by, other transition metal ions of similar size and charge e.g Zn^{2+},Cu^{2+} and Fe^{3+} (Kustin and Macara 1982). The redox behaviour of the element during its conversion from cation to anion at physiological pH may be crucial for its biological role (Kustin and Macara 1982).

Vanadium Toxicity

Vanadium is present in a porphyrin in plant tissue and has accumulated in relatively high concentrations in fossil fuels. Combustion of these fuels has released particulate vanadium into the atmosphere. If the particles are inhaled, the vanadium comes into direct contact with cells (Ramasarma and Crane 1981; Carlton et al 1982). Effects of exposure manifest themselves by

respiratory tract irritation including asthma, bronchitis and pneumonia (Ramasarma and Crane 1981; Musk and Tees 1982). Excess dietary vanadium (25ppm) was toxic to rats and chicks (Hathcock et al 1964) and 100-200ppm ($3x10^{-3}$M) caused a rise in blood pressure in rats and dogs (Steffen et al 1981). It had a variety of neurotoxic effects (Donaldson and LaBella 1983) and these have been suggested to be due to the generation of active superoxide anions and hydroxyl radicals which affect neurotransmitter-receptor binding. The auto-oxidation of catecholamines and the subsequent release of cytotoxic products may also be responsible for the toxic effects seen (Donaldson and LaBella 1983).

High blood levels of vanadium have been found in patients suffering from manic depression where the sodium pump activity is impaired, which may render them more vulnerable to vanadate (Naylor 1983). Some cancer patients have also been shown to have increased blood vanadate levels (Agarwal and Sant 1978).

Vanadium has been claimed to have a therapeutic effect in pharmacological doses and has been used in the treatment of anaemia, tuberculosis, and certain other chronic diseases. This was especially popular at the end of the last century in France (Falkner-Hudson 1964). It has been shown to have some anti-spirochaetal properties, and was used for the treatment of syphillis (see Falkner-Hudson 1964).

At pharmacological levels vanadium decreased cholesterol synthesis in humans and animals (Underwood 1977), accompanying which a decrease in plasma phospholipid and cholesterol, and reduced aortic cholesterol concentrations were noted. In older individuals, and those suffering from hypercholesteremia or

ischemic heart disease this vanadium effect was not apparent (Sommerville and Davis 1962; Dimond et al 1963). In older rats the inhibition of cholesterol synthesis could be seen in vitro, but not in vivo. The site of inhibition has been shown to be the microsomal squalene synthetase (Azarnoff et al 1961).

Accumulation

The vanadium content of sea water is very low (0.0003-0.003 ppm, 50nm, Goldschmidt 1954). Most common invertebrates contain 1-3ppm (dry weight) and some can accumulate large amounts in blood and tissues (Henze 1911; Bertrand 1950) Many ascidians, some holothurians and one mollusc Pleurobranchus plumula, accumulate up to 1900 ppm (Bertrand 1950). Amanita muscaria, the fly agaric, accumulates vanadium and has been shown to have 180 ppm dry weight. No other fungus has been shown to have this capacity (Schwarz and Milne 1971; Macara 1980).

In Ascidia nigra the blood vanadium concentration can be as high as 1M. No other organism is known to have such an efficient accumulation (Carlson 1976). The reduced V^{3+} is accumulated in vacuoles in specialised blood cells, or vanadocytes, which are green in colour. The vanadium is possibly stabilised by chelation to small molecules since the vacuoles are not acidic (Kustin and Macara 1982). They do not have a respiratory function, (Macara et al 1979) but it has been postulated that vanadium may be important in the formation of the tunic, in which exocytosis of the vacuolar contents may be followed by a redox reaction to generate cross links with tunic components (Macara 1980; Kustin and Macara 1982). It has also been proposed that

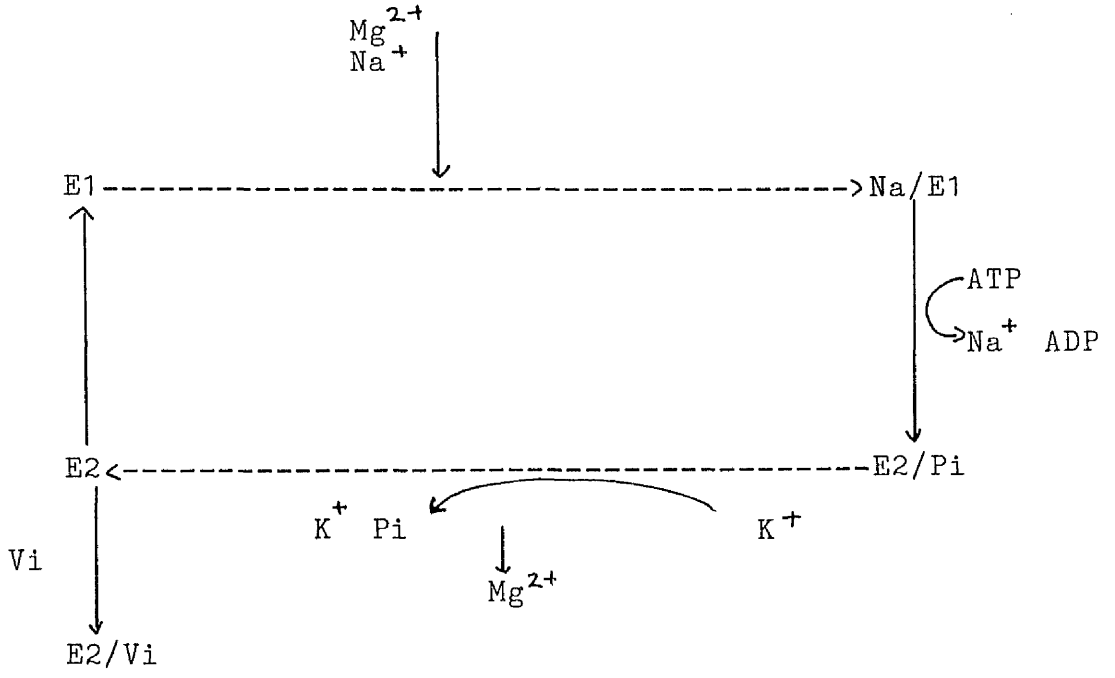
vanadium may have anti-microbial properties (Rowley 1983).

In mammalian tissue, vanadium concentration has been found to be $1x10^{-7}$ - $1x10^{-6}$M (Underwood 1977; Cantley et al 1978a). Even at these concentrations it can be shown to have a variety of effects on physiological function, as will be discussed later.

A large number of different enzymes have been shown to be affected by vanadate in vitro. This effect is thought to be due to the vanadate ion behaving as a phosphate analogue. As already discussed vanadate and phosphate have very similar structures (see above). Table 2 outlines the enzymes that are inhibited (or stimulated) by vanadate (or other forms of vanadium).

The analogy between phosphate and vanadate is exemplified by its inhibition of the Na/K ATPase

OUTSIDE



INSIDE

Table 2

Effect of Vanadate on various enzymes.

Enzyme	Ki M [vanadate]	I/S	Ref
Erythrocyte Na/K ATPase	$1x10^{-6}$M	I	1
Ca^{2+}ATPase (SR)	$1x10^{-6}$M	I	2
Ca^{2+}ATPase (membrane)	$5X\ 10^{-6}$M	I	3,4
K^{+}/H^{+} (gastric mucosa)	___	I	2
Ca^{2+}ATPase (Squiud axon)	$1x10^{-3}$M	I	5
K^{+}ATPase E.coli	___	I	2
ATPase S.cervisiae	$7X10^{-6}$M	I	6
ATPase N.crassa	$1x10^{-6}$M	I	7
ATPase (plant plasma membrane)	$2.5X10^{-5}$M	I	8
K^{+}/H^{+}ATPase Vicia faba	$1x10^{-6}$M	I	9
Myosin ATPase	$5x10^{-3}$M	I	10
Dynein ATPase	$1x10^{-7}$M	I	11
Adenylate cyclase	$1x10^{-4}$M	S	12,13,14
Ribonuclease	$5X10^{-5}$M	I	15
Phosphofrucktokinase	$1x10^{-6}$M (D)	I	16
Adenylate kinase	$1x10^{-6}$M (D)	I	17
Acid phophatase			
human liver	$2X10^{-7}$M	I	18
human astrocytoma	$5X10^{-5}$M	I	19
wheat germ	$7X10^{-6}$M	I	18
lichen	___	I	20
Alkaline phosphatase			
E.coli	$2X10^{-5}$M	I	21
	$4X10^{-7}$M(E)	I	21
human liver, intestine and kidney	$1x10^{-6}$M	I	22
HMG-CoA reductase	$1x10^{-3}$-10^{-2}M(E)	I	23
NAD(P)H oxidase			
ER,microsome, golgi	$3x10^{-4}$M (D)	S	24
liver cell membrane	$1x10^{-3}$M	S	25

Gluteraldehyde-3-phosphate dehydrogenase	$2X10^{-2}M$	I	17
Succinate oxidase	___	I	26
Glutamate oxidase	___	I	27
Phosphoglycomutase	$1x10^{-3}M$	S	28
Carboxypeptidase A	$1x10^{-6}M$ (E)	S	29
Chain intiation rabbit reticulocyte lysate	$2X10^{-5}M$	I	30
Chain elongation rabbit reticulocyte lysate	$5X10^{-5}M$	I	30
DNA polymerase	$1x10^{-5}M$	I	31
Terminal deoxynucleotidyl transferase	$1x10^{-5}M$	I	31
DNA polymerase 1 E.coli	$5X10^{-5}M$	I	31

D = decavanadate

E = vanadyl (V^{4+})

1) Cantley et al 1977

2) O'Neal et al 1979

3) Bond and Hudgins 1980

4) Barrabin et al 1980

5) Dipolo et al 1979

6) Dufour et al 1980

7) Bowman and Slayman 1979

8) Sze 1983

9) Gepstein et al 1982/83

10) Goodno 1983

11) Gibbons et al 1978

12) Schwabe et al 1979

13) Krivanek 1981

14) Ajtai et al 1983

15) Lindquist et al 1973

16) Choate and Mansour 1979

17) DeMaster and Mitchell 1973

18) Van Etten et al 1974

19) Leis and Kaplan 1982

20) Le Sueur and Packett 1980

25) Erdmann et al 1979

26) Aiyar and Sreenivasan 1961

27) Byczkowski et al 1979

28) Layne and Najjar 1979

21) Lopez et al 1976

22) Seargeant and Stinson 1978

23) Menon et al 1980

24) Ramasarma et al 1981

29) DeKoch et al 1974

30) Ranu 1983

31) Sabbioni et al 1983

The Na/K ATPase can exist in two forms E1 and E2 (see diagram above). E1 binds Na^+, Mg^{2+} and ATP promoting the formation of an E2-P intermediate following the hydrolysis of ATP. The terminal phosphate is transferred to an aspartyl residue of the enzyme. K^+ binding promotes the breakdown of the phosphate intermediate and Mg^{2+} and phosphate are released in the rate determining step to E1 (Kustin and Macara 1982). As indicated in the diagram vanadate binds to the enzyme in the E2 state, binding to the cytoplasmic side of the molecule and preventing the return to the E1 conformation.

Vanadate forms ternary complexes with myosin and dynein ATPases, acting again as a phosphate analogue and inhibiting the action of the enzymes (Kustin and Macara 1982).

myosin
dynein - ADP - vanadate

In contrast to the inhibitory effects of vanadate on the ATPases and other enzymes (see Table 2), the ion stimulates the adenylate cyclase of a number of cell types (Schwabe et al 1979; Krivanek 1981; Ajtai et al 1983). Mammalian adenylate cyclase consists of three subunits : the catalytic unit, the prostaglandin hormone regulatory unit and the GTP binding protein (a GTP hydrolase; Lester et al 1982). Vanadate appears to act through the G protein which itself acts as a link between the

other two subunits. When GTP is bound it activates the adenylate cyclase. When GDP is bound there is no activation. The G protein hydrolyses GTP $\longrightarrow$ GDP + Pi.

If vanadate is bound to the E-GDP it forms a stable ternary complex E- GDP- Vanadate which behaves as if GTP were bound (Kustin and Macara 1982; Ajtai et al 1983). Thus the initially surprising activation of adenylate cyclase by vanadate can be explained in terms similar to that for the mechanism of inhibition of the myosin and dynein ATPases.

Reversal of Vanadate Action

Reversal of vanadate inhibition of the Na/K ATPase and a variety of other enzymes was observed with addition of norepinephrin (arterenol) and other catechol compounds, through vanadate reduction and complex formation (Josephson and Cantley 1977; Cantley et al 1978b; Adam-Vizi et al 1980). Ascorbic acid and glutathione have also been shown to reverse vanadate effects (Mitchell and Floyd 1954; Grantham and Glynn 1979), of which the latter has been shown to be important in the cytoplasmic reduction of $V^{5+} \longrightarrow V^{4+}$ (Macara 1980; Macara et al 1980).

In erythrocytes V^{4+} binds to haemoglobin (Cantley and Aisen 1979) and in adipocytes it appears largely to form a complex with glutathione (Degani et al 1981). This reduction could explain the resistance of Na/K ATPase to vanadate in intact cells (Macara et al 1980). Additions of high concentrations of vanadate may allow cytoplasmic vanadate to accumulate transiently before being complexed to small molecules and protein (Macara et al 1980).

V^{4+} has less effect on Na/K ATPase and other enzyme systems, however its cytoplasmic effects are not known. It has been reported that V^{4+} does have an inhibitory effect on some isolated enzymes in vitro (DeKoch et al 1974; Van Etten et al 1974; Lopez et al 1976; Menon et al 1980). Large concentrations of vanadate may exert an effect by oxidising cytoplasmic glutathione (Macara et al 1980).

A variety of chelating agents have been shown to reduce the amount of cell-bound vanadium in whole rats, e.g. Desferrioxamine B (Desferal) and Ca-Na_3 - diethylene triamine pentaacetic acid (DTPA) (Hansen et al 1982).

A variety of non-enzymic effects of vanadate have been described and these can be attributed to the redox properties of the ion in aqueous solution (see Table 1).

Vanadate and vanadyl ions can abstract sulphur from cysteine producing H_2S, NH_3 and pyruvate in the presence of pyridoxal phosphate (Bergel et al 1958). In the presence of pyridoxal phosphate it has been shown that vanadate can catalyse the non-enzymic transamination of 4,5 dioxovalerate and alanine (Meisch et al 1978). Vanadate and vanadyl ions stimulate the transamination of 4,5 dioxovalerate-alanine to delta aminolevulinate-pyruvate (Ramasarma and Crane 1981).

Vanadate forms complexes with catechols, (see section on vanadate reversibility) and can oxidise norepinephrin and epinephrin (Cantley et al 1978b; Quist and Hokin 1978). The reaction removes the active vanadate species, but also destroys the catechol non-enzymically (Cantley et al 1978b).

As well as stimulating NAD(P)H oxidase, vanadate has been shown to oxidise NAD(P)H non-enzymically, H_2O_2, O_2^- and $OH^\cdot$ radicals are formed (Ramasarma et al 1981) and these may account for some of the diverse biochemical effects of vanadate. Vanadyl sulphate has been shown to reduce Cytochrome C (Ramasarma et al 1981) and oxidation reduction between vanadyl ions and H_2O_2 can catalyse non-enzymatic hydrolysis of ATP (Wolterman et al 1974). This is interesting in view of the existence of cytoplasmic vanadium as vanadium 4+ and an ATP-V^{4+} complex. This complex could promote ATP hydrolysis and oxidised vanadate then inhibit ATPases so that the oxidation-reduction status of vanadium could have a regulatory role on ATP concentration (Ramasarma and Crane 1981).

Effects of Vanadate on Isolated Tissues and Organs

The effects of vanadate on a variety of enzymes, already outlined in Table 2 , could account for a number of actions of the ion observed on isolated tissues and organs. These include the increased force of contraction (positive inotropic effect) of isolated cardiac muscle of rat, dog, cat and rabbit (Hackbarth et al 1978; Grupp et al 1980), and the negative inotropic effect on the left atrial muscle of cat and guinea pig heart (Borchard et al 1979). These effects have been proposed to be mediated through vanadate inhibition of Ca^{2+} ATPase of the sarcoplasmic reticulum (Kustin and Macara 1982; Gesser and Jorgensen 1983). Isolated smooth muscle preparations showed different sensitivities to induction of contraction by vanadate. This was also proposed to be via a Ca^{2+} involvement (Fox et al 1983;

Naylor and Sparrow 1983). In support of this there is some evidence to show that vanadate increased the cytosolic Ca^{2+} concentration in skeletal muscle (Clausen et al 1981).

Vanadate is a powerful natriuretic and diuretic (Balfour et al 1978), and decreases the fluid reabsorption in superficial proximal tubules (Higashi and Bello-Reuss 1980) and inhibits PAH accumulation in cortical slices of isolated kidney (Edwards and Grantham 1983). These effects are thought to be due to the inhibition of the Na/K ATPase (Edwards and Grantham 1983). The inhibition of renin secretion from rat kidney slices has been proposed to be due to vanadate-mediated changes in the internal calcium concentration (Churchill and Churchill 1980).

Vanadate inhibited urinary acidification in turtle bladder (Arruda et al 1981). This was proposed to be mediated via inhibition of a proton translocating ATPase (Arruda et al 1981). In toad bladder it was proposed that the stimulation of adenylate cyclase was reponsible for the alteration of water transport upon administration of vanadate (Arruda and Westenfelder 1983). Similarly the stimulation of secretion of chloride ions by isolated rabbit colonic epithelium was thought to be due to the vanadate activation of adenylate cyclase (Hatch et al 1983).

Growth Factor-Like Effects Of Vanadate

A number of the reported actions of vanadate in intact cells resemble the effects of certain of the polypeptide growth factors. For instance Cassel et al (1984) have recently reported that vanadate ($1x10^{-4}M$) stimulated the Na^+/H^+ exchange activity in A431 cells in a manner similar to that of EGF (and see below).

DNA Synthesis

Vanadate was shown to be mitogenic for a subpopulation of T cells (Ramanadham and Kern 1983). Vanadate has also been shown to stimulate quiescent Swiss mouse 3T3 cells in serum free medium (Smith 1983). In these cells it appeared that vanadate stimulated, rather than inhibited, the Na/K ATPase. This is interesting in view of the fact that stimulation of the Na/K ATPase in this cell line is known to stimulate DNA synthesis (Rozengurt 1980). The vanadate acted synergistically with 1%-4% serum and there was a large co-operative effect with vanadate and insulin in serum free medium. This was thought to be due to the stimulation of the cells, rather than a chemical modification of the insulin (Smith 1983). DNA synthesis in quiescent human fibroblasts was also stimulated by vanadate, where again it was shown to have a synergistic effect with EGF (Carpenter 1981). Colchicine and other anti-microtubule agents had effects on DNA synthesis similar to those of vanadate and acted synergistically with some growth factors (Friedkin et al 1979 and see discussion). Taxol almost totally prevented the stimulation of DNA synthesis by vanadate (Smith 1983).

Insulin-Like Effects

A number of effects of vanadium can be regarded as insulin-mimetic on the whole cell, including stimulation of glucose transport and sodium pump (Clausen et al 1979) and stimulation of DNA synthesis (Fitzgerald and Chasteen 1974; Hori and Oka 1980), positive inotropic effects on cardiac muscle (Grupp et al 1979), inhibition of protein degradation (Seglen and Gordon 1981) and mouse cell hyperpolarisation (Zemkova et al 1982). In muscle and

fat cells the rate of glucose analogue uptake and glucose oxidation increase upon vanadate treatment and lipolysis occurs in a manner similar to insulin treated cells (Dubyak and Kleinzeller 1980; Shechter and Karlish 1980; Degani et al 1981). Vanadate has also been shown to enhance rat adipocyte glycogen synthase and the degree of phosphorylation of the 93Kd subunit of the insulin receptor, specifically on tyrosine residues, as does insulin (Tamura et al 1983).

Phosphorylation

Reversible phosphorylation of proteins has come to be accepted as a major regulatory feature of protein function in eukaryotes (See Foulkes 1983 and refs. therein).

Of the phosphoamino acids found in phosphoproteins, phosphoserine is the most abundant (Taborsky 1974). Some transforming gene products have been shown to possess tyrosine specific protein kinase activity (Foulkes 1983 and refs therein), and some transformed cells have higher levels of phosphotyrosine than their normal counterparts (Erickson et al 1980). Growth factor stimulation of cells results in the phosphorylation of specific receptors as well as a spectrum of substrates (Ushiro and Cohen 1980; Ek et al 1982; Zick et al 1983). It is interesting to note that in human astrocytoma phophotyrosine phosphatases were shown to be inhibited by vanadate ($5x10^{-5}$ M; Leis and Kaplan 1982), and in Raji human lymphoblastoid cell membranes vanadate stimulated tyrosine phosphorylation of two proteins ($1x10^{-6}$M; Earp et al 1983). Tyrosine phosphorylation of the insulin receptor was enhanced in vanadate treated preparations ($1x10^{-3}$ M; Tamura et al 1983). The levels of

phosphorylation of the pp60^{src} kinase isolated from RSV-transformed chick embryo fibroblasts were increased after incubation of the intact cells in vanadate (Brown and Gordon 1984).

Vanadate And Possible Interactions With Intermediate Filaments

Vanadate has been shown to disrupt IF distribution, leaving mts intact within the cytoplasm of single cells and virus-induced syncytia (Wang and Choppin 1981).

Vanadate also inhibits the aggregation of trypsin-suspended BHK-21 cells (Edwards 1981) to an extent similar to the inhibition of aggregation observed with colchicine, approximately 80% inhibition when compared to controls over a period of one hour (Waddell et al 1974). The mechanism of inhibition of aggregation by vanadate is unknown, but one possibility involves the attachment of IFs to the inner face of the plasma membrane (Edwards and Dysart 1980). Alternative theories are considered in the discussion.

In view of the effects of vanadate on IFs it is appropriate to review here some aspects of IF biology.

INTERMEDIATE FILAMENTS

Diversity

Intermediate filaments, first described as such by Ishikawa et al (1968), were so called because they were intermediate in diameter between the thick myosin filaments (15nm) and the thin microfilaments (6nm) in the developing myoblasts studied.

The two major techniques developed to identify these elements, namely biochemical analysis and immunohistochemistry, have led to the understanding that there are five classes of filament (see Table 3).

Despite their different biochemical and immunofluorescent characteristics intermediate filament proteins have a large degree of structural homology and similar physical properties. They are all approximately 10nm in diameter and frequently appear to have a less dense core which gives them a tubular appearance (Zackroff et al 1981). They are relatively insoluble at physiological ionic strength and pH, and this property has been exploited for their extraction and isolation from cultured cells (Brown et al 1976; for a more detailed discussion of this see Anderton 1981). For details of structure, assembly and other aspects of IF biology see reviews by Lazarides 1980; Anderton 1981; Osborn et al 1981; Weber and Osborn 1981; Fuchs and Hanukoglu 1983.

Table 3

Summary of Intermediate Filament proteins and their distribution.

Protein	MW (Kd)	Distribution	References
Cytokeratin	40-70 19 different proteins so far identified	Epithelial cells	Anderton 1981 Moll et al 1982
Desmin/ Skeletin	50-55	Muscle cells Z-lines of skeletal muscle Intercalated discs of Cardiac muscle	Anderton 1981 Small and Sobieszcek 1971 Lazarides 1980 Lazarides and Balzer 1978 Campbell et al 1979
Vimentin	58	Mesenchymal cells immature neurones and glia and cells adapted to culture	Anderton 1981 Franke et al 1979a
Glial Fibrilliary Acidic Protein (GFAP)	49-54	Astrocytes Glia	Weber and Osborn 1981 Anderton 1981
Neurofilaments	68,150,200 150,160 60,200	Mammalian Neurones Myxicola Neurones Squid axons	Schlaepfer 1977 Gilbert 1975 Lasek et al 1979

Intermediate Filament displays in cultured cells

Immunofluorescent studies have provided beautiful visualisations of the IF distribution within the cell (e.g. Franke et al 1978a and 1978b; Frank et al 1982). In cultured fibroblasts the vimentin filaments form filamentous networks that radiate through out the cell (Gordon et al 1978). The filaments are particularly abundant close to the nucleus and do not enter motile areas of the cell (Weber and Osborn 1981). The vimentin fibres aggregate to form perinuclear whorls upon treatment with colchicine and other anti-mitotic drugs or low temperatures (Bennett et al 1978 and see below). These perinuclear whorls are also observed by Electron Microscopy (Ishikawa et al 1968; Goldman and Knipe 1973).

Epithelial cells in culture contain cytokeratins and vimentin, the latter perhaps as an adaptation to culture (see above). The immunofluorescence studies with antibodies to cytokeratin have revealed a wavy fibrillar network that is often branched (Osborne et al 1977; Franke et al 1978a; Sun et al 1979). These fibres have a predominantly radial arrangment and like vimentin, do not enter motile areas of the cell, although short fragments can often be detected near the cellular margins (Osborn et al 1977; Franke et al 1978b). In EM some of these cytokeratin filaments appear to be associated with the desmosomes (e.g. Franke et al 1979b). The vimentin filaments have a different distribution within the cells, as visualised by double immunofluorescence (Osborn et al 1980). As explained below, the different proteins can readily be separated by the different sensitivity of their displays to colchicine. In addition to vimentin, desmin and GFAP filaments also form juxtanuclear

aggregates upon treatment with colchicine and Vinca alkaloids (Ishikawa et al 1968; Osborn et al 1977; Gordon et al 1978; Starger et al 1978). Juxtanuclear aggregates of all IF classes can be obtained by micro-injection of the appropriate antibodies to the filament proteins (Eckert and Daley 1981; Gawlitta et al 1981; Klymkowsky 1981; Lin and Feramisco 1981).

Other treatments that have been shown to disrupt the IF displays in cells include cold shock in epithelial cells (Schliwa and Euteneuer 1979), heat shock in BHK cells (Falkner et al 1981), vanadate ions in virus induced syncytia and single BHK cells (Wang and Choppin 1981) and activation of a temperature sensitive Src gene in BHK cells (Ball and Singer 1981). Changes in filament distribution also occur during mitosis (e.g. Blose 1981; Horrowitz et al 1981; Blose and Bushnell 1982; Franke et al 1982; Lane et al 1982).

Intermediate Filament Function

The function of IFs remains elusive but there is much circumstantial evidence that the filaments are associated with various cytoplasmic organelles. For example:- centrioles (Starger et al 1978; Aubin et al 1980) and mitochondria (David-Ferreira and David-Ferreira 1980; Mose-Larsen et al 1982), microtubules (Goldman and Knipe 1972; Geiger and Singer 1980; Ball and Singer 1981). This has led to the proposition that the filaments play an important role in the mechanical integration of various cytoplasmic organelles (Wang and Goldman 1978; Lazarides 1980; Wang et al 1981).

Use of IF-disrupting agents (e.g. vanadate ions and micro-injection of specific antibodies) had been shown to disrupt organisation of some organelles (Wang and Choppin 1981). The effect of microtubular poisons on IFs has been proposed to be due to loss of interaction of IF with Mts (Goldman et al 1978). Addition of taxol caused the Mts to bundle and the If followed this rearrangment even in the presence of Mt poisons (Geuens et al 1983; Maro et al 1983).

The nature of the interaction between IF and Mts is not known. Links have been seen between the two structures in freeze-fracture and thin sections (Franke et al 1981a; Hirokawa 1982). Micro-injection of anti-tubulin antibodies has been shown to disrupt IF displays but not those of Mts (Blose et al 1984). It has been proposed by these authors that these antibodies were acting at a site of Mt-IF interaction.

The striking perinuclear distribution of the filaments has led to the proposal that they may function in nuclear anchorage (Lehto et al 1978) and there have been suggestions that there are IF organising centres at the nucleus (Eckert et al 1981;1982).

IF may be involved in cell-cell adhesion as IF appear to be associated with mf bundles (Singer et al 1981) at sites where alpha actinin is concentrated. An association between skeletin and alpha actinin in bovine heart Purkinje fibres (Kjorell and Thornell 1982) has been reported.

Desmin, present in embryonic striated muscle, localises, as the muscle develops, at the Z lines, cell-cell junctions and where Z lines appose the plasmamembrane (at intercalated discs in cardiac muscle and dense bodies- Z line analogues in smooth

muscle (Cooke 1976; Lazarides 1978; Lazarides and Hubbard 1978).

Desmosomes (Maculae adherentes) have distinctive morphological and biochemical characteristics (Skerrow and Matoltsy 1974), and are highly conserved structures (Cowin and Garrod 1983). The typical organisation is characterised by an extracellular midline (stratum centrale) the two plasmamembranes and the two plaques at which cytoplasmic bundles of IF anchor. Desmosomes are typical of epithelial cells and it has been shown, using antibodies to the desmosomal plaque proteins (desmoplakins) and cytokeratins, that the IF are specifically attached to the desmosomal plaques in epithelial cells (Franke et al 1981b; Geiger et al 1983). Cardiac myocytes, which do not contain cytokeratins are connected by desmosomal structures containing similar proteins to the epidermal desmoplakins and the IF protein attached to these plaques has been identified desmin (Kartenbeck et al 1983; Tokuyasu et al 1983). Desmoplakins have also been shown to occur in meningiomas, intracranial tumours, that express IF of the vimentin type. In the cells of these tumours vimentin has been shown to attach to the desmosomal plaques (Kartenbeck 1984). This possible role of IF in cell to cell adhesion is interesting in view of the work of Edwards and Dysart (1980). Extracted cytoskeletons of confluent monolayers of BHK-21 were prepared and shown to consist of nuclear envelopes linked by IF bundles. Use of a micromanipulator showed that tension could be transmitted from cell to cell through many cell lengths, indicating that filaments must be attached to the cell surface at least at points of intercellular adhesion. The continuity within the cells suggests that IF bundles of vimentin and desmin could form continuous extension resisting structures (Cooke 1976;

Edwards and Dysart 1980). This asssumes that the intercellular adhesion was not an artifact of the preparation of these cytoskeletal structures.

Other possible functions of IF that have been proposed include maintenance of cell shape (Goldman et al 1979; Dabike et al 1981; Duffy et al 1982; Jones et al 1982) and motility (Duffy et al 1982; Jones et al 1982). Micro-injection of antibodies to IF left Mts intact. Saltatory movement and locomotion and division were observed for at least 20-30 hours after injection (Gawlitta et al 1981). The cells of the rapidly dividing inner cell mass (ICM) of the mouse blastocyst are devoid of filaments - yet survive (Jackson et al 1980; Paulin et al 1980).

It seeems possible that the filaments function at a tissue level, possibly to maintain the mechanical integrity of the tissue to function in vivo. Their function remains elusive with present methods of investigation.

AIMS OF RESEARCH

Edwards (1981) showed that vanadate ions inhibited the aggregation of BHK-21 cells to an extent similar to that of colchicine and other microtubule poisons (Waddell et al 1974). In view of the many effects of vanadate described in the literature, especially the redistribution of IF (Wang and Choppin 1981) it was clear that more information about its effects on cells in culture would be needed before the mechanism by which it inhibits cell aggregation could be understood.

I therefore examined various interactions of vanadate with cultured cells. This investigation fell into 3 broad categories of approach:-

1.) The effects of vanadate on growth and locomotion to determine whether or not it was non-selectively inhibiting a wide range of cellular activities and functions. Attention was paid to whether its actions were detectable in the long or short term.

2.) The uptake of vanadate, since if vanadate does not enter live cells this would greatly restrict possible sites of inhibition.

3.) The effects of vanadate on cell spreading, initially to look for inhibition of cell-substrate adhesion to parallel that already observed for cell-cell adhesion. Since the inhibition of spreading was demonstrated to be reversible, the vanadate ions were obviously not toxic to the cells under these conditions. This work was developed to test possible mechanisms for this inhibition.

MATERIALS AND METHODS

MATERIALS

Media

Media used throughout this work are specified below. All solutions were made up in reverse osmosis water further purified by adsorption and ion-exchange and, where necessary, sterilized by autoclaving and subsequently stored at 4°C. All chemicals used were of Analar grade where possible.

Hepes Saline (HS)

For 5L	
NaCl	40g
KCl	2g
D-glucose	5g
Phenol red (1/2%)	10ml
Hepes	11.92g
pH adjusted to 7.5.	

Hanks Hepes (HH)

For 5L	
NaCl	40g
KCl	2g
D-glucose	5g
Phenol red (1/2%)	10ml
Hepes	11.92g
$CaCl_2.2H_2O$	0.93g
$MgCl_2.6H_2O$	1g
pH adjusted to 7.5	

Hepes is N-2-hydroxethylpiperazine-N'-2-ethane sulphonic acid (Cambridge Research Biochemicals).

Versene (V)

For 5L	
NaCl	40g
KCl	1g
Phenol red (1/2%)	15ml
Na_2HPO_4	5.75g
KH_2PO_4	1g
Na_2EDTA	1g

pH adjusted to 7.4

Tris Saline

For 1L	
Tris-base	3.02g
NaCl	8.19g
KCl	0.37g
Na_2HPO_4	0.01g

c
Tryptose Phosphate Broth (TPB)

147.5g TPB (Difco) dissolved in 5L double-deionised water and pH adusted to 7.4.

Trypsin

0.25% trypsin (Difco 1:250) made up in Tris Saline was obtained from the Dept. Virology, University of Glasgow. Activity approximately 1000 BAEE (N-Benzoyl-l-arginine ethyl ester) units. Used diluted 1 in 5 in versene.

Bicarbonate Buffered Eagles Medium (ECT)

135ml Sterile water
16ml x10 concentrate Glasgow modified Eagles Medium (Flow)
6ml 7.5% $NaHCO_3$
5ml GPSA (Glutamine (200mM), Penicillin (200 000 units/ml), Streptomycin (200 000 units/ml), Amphotericin B: Flow
20ml Calf Serum
20ml TPB

Cells grown in ECT were equilibrated with a gas phase of 95% air, 5% CO_2 Mixture, for buffering purposes.

Hepes Buffered Eagles Medium (HEFT)

180ml	20mM Hepes water
20ml	x10 concentrate Glasgow Modified Eagles
5ml	GPSA
20ml	Calf serum
20ml	TPB

Hams F10

180ml	20mM Hepes water	5ml	GPSA
20ml	x10 concentrate Hams F10 (Flow)	20ml	TPB
1ml	7.5% $NaHCO_3$	20ml	Foetal calf serum

Phosphate Buffered Saline (PBSa)

NaCl	0.17M	
KCL	3.4mM	
Na_2HPO_4	10mM	
KH_2PO_4	1.8mM	pH adjusted to 7.2.

Stock Solutions

Wherever possible 40x concentrated stocks were prepared and 25µl stock added per ml of medium as required. Analar grade reagents were used.

Ammonium metavanadate (NH_4VO_3) (Sigma)
4mM solution prepared in HS at pH 8. This was further diluted with HS, pH 8, as required.

Colchicine (Sigma)

4mM solution prepared in HH.

Arterenol (Nor-epinephrin) (Sigma)

40mM solution prepared by dissolving 67.3mg arterenol in 10ml HH. This was acidified with 0.1ml 1N HCl to allow the arterenol to dissolve.

Ouabain (Sigma)

10mM solution prepared in HH. Due to the relative insolubility of ouabain this 10x concentrate was used.

dibutyryl cyclic AMP (db cAMP) (Sigma)

10mM solution was made in HH. Again due to its relative insolubility a 10x concentrate was used.

Isobutyryl-1-Methyl Xanthine (IBMX) (Sigma)

100x concentrate was used by preparing a 100mM solution in DMSO.

Fibronectin

Fibronectin was isolated from calf serum on a gelatin-Sepharose column, using the method of Engvall and Ruoslahti (1977). Stocks at a concentration of approximately 1mg/ml in 8M Urea were stored at 4°C. Samples were run on SDS-PAGE to assess the purity of the FN used.

Cytoskeleton Extraction Reagents

HH

HH + 2mM Phenylmethylsulphonyl fluoride (PMSF) (Sigma)
1mg/ml p-Tosyl-l-arginine methyl ester-HCl (TAME) (Sigma)

HH + 1% Triton X-100 (Polyethyleneglycol (9-10) p-t-octylphenol)
PMSF and TAME (Sigma)

HS + 0.5% Triton X-100
10mM $MgCl_2$
0.1mg/ml Deoxyribonuclease 1 (Sigma) specific activity 1500 Kunitz units/mg protein

HH + 1.5M KCl

Polyacrylamide Gel Electophoresis (PAGE) reagents

Materials and methods used as described by Laemmli (1970). All reagents were specially purified for electrophoresis. Acrylamide and bisacrylamide from Koch-Light, Sodium dodecyl sulphate (SDS) and Kenacid blue R (C.1.42660) from BDH, Ammonium persulphate, bromophenol blue and N,N,N',N'-tetramethylethylenediamine (TEMED) from Bio-Rad and Tris(Hydroxymethyl)aminomethane (Tris) and glycine from Sigma.

Molecular Weight Standards

The following proteins were used at a concentration of 1mg/ml.

Fibronectin	220Kd	Catalase	60Kd
Beta galactosidase	116Kd	Ovalbumin	45Kd
Phosphorylase A	95Kd	Concanavalin A	22Kd

Electron Microscopy

The following solutions were prepared for processing cells for Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

0.2M Sorensens' phosphate buffer at pH 7.4, 19ml 0.2M KH_2PO_4 + 81ml 0.2M Na_2HPO_4

Glutaraldehyde fixative, 1ml 25% Glutaraldehyde (EM Scope)
9ml 0.2M phosphate buffer

Osmium fixative, 1% OsO_4 in 0.2M phosphate buffer

For TEM, Araldite resin (Agar Aids) was used

10ml Araldite

10ml Dodecyl Succinic Anhydride (DDSA)

1ml Dibutyl Phthalate

0.5ml 2,4,6 Tri(dimethylaminomethyl) Phenol (DMP 30)

were added and mixed well.

Stains

Uranyl Acetate, a saturated solution of uranyl acetate in 50% methanol was prepared.

Lead Citrate, 1.33g $Pb(NO_3)_2$
1.76g $Na_3(C_6H_5O_7).2H_2O$

30ml double-deionised water was added and the mixture shaken vigorously for 5 minutes and left for 30 minutes with occasional gentle agitation. 8ml 1N NaOH was added and 12ml double deionised water added to bring total volume to 50ml.

Radiochemicals

The following radiochemicals were obtained from Amersham International plc. Amersham UK.

^{32}P (carrier free)	specific activity	5Ci/mMol
^{35}S Methionine		>800Ci/mMol
^{3}H Thymidine		25Ci/mMol
^{48}V (acidic $VOCL_2$)		200mCi/mg V

Scintillation Fluid

Pico-Fluor-15 obtained from United Technologies Packard, and dispensed in 10ml aliquots. For the ^{32}P-phosphate a 0.4% solution of PPO (2,5 Diphenyloxazole) (Koch-Light) in toluene was used.

Antibodies

Monoclonal mouse anti-vimentin (Labsystems) was used at a 1in 10 dilution.

Monoclonal mouse anti-tubulin (Amersham International) was used at a 1 in 500 dilution.

Fluorochromes

Rhodamine-Phalloidin (Molecular Probes Inc.) was used to visualise the actin containing stress fibres of the cells. 25µl of the Rhodamine-Phalloidin solution (in methanol) was placed in an Ependorf tube. Once the alcohol had evaporated 500µl PBSa was added and this was then applied to the coverslips.

Texas red conjugated sheep anti-mouse immunoglobulin (Amersham International) was used at a 1 in 25 dilution.

Hoechst 33258 was used as a DNA fluorochrome, at a 1 in 1000 dilution.

Photographic Materials

Pan-X film used for phase and bright field optics, developer 50% D76, fixer 30% Amfix (Kodak). AHU film used for photographing gels, developer 100% D19, fixer 30% Amfix (Kodak). XP1-400 film for immunofluorescence , developed using XP1-400 kit (Ilford). Autoradiographs of ^{3}H thymidine treated cells were made using Ilford Nuclear Emulsion, developer 100% D19, fixer 30% Amfix (Kodak). For printing the paper used was Kodabrome IIRC grades 2 and 3, developer 10% DPC, fixer 12% Amfix (Kodak).

Siliconised Flasks

25ml conical flasks were cleaned and dried and filled with 1% silicone DC1107 at a 1% concentration in ethyl acetate. After 30 minutes the silicone fluid was poured out, and the flasks baked at 160°C for 2 hours and sterilized before use.

Tissue Culture Vessels

Plastic 90mm and 35mm diameter Petri Dishes (Sterilin),
$25cm^2$ and $75cm^2$ plastic flasks (Falcon, Sterilin).
Sterile glass Winchester bottles for roller cultures.

Cells

Cells used were BHK-21 C13 (Stoker and MacPherson 1964) and their transformed derivatives, Polyoma transformed Py, and Avian Sarcoma Virus, Schmitt-Ruppin variant (ASV) transformed cells (MacPherson 1965).

METHODS

Cell Harvesting

Cells for all experiments were grown in bicarbonate-buffered ECT, in tissue culture grade plastic flasks or sterile glass bottles. Cells were grown in this manner until approximately confluent, usually 1-3 days depending on inital number seeded.

Preparation of cell suspensions

The cell monolayers were washed twice with HS, and trypsin/versene (1 in 5 dilution) was added for 1 minute. Excess fluid was poured off and the cells left for a further 4 minutes during which time the flasks were gently agitated to aid the detachment of the cells from the substrate. The trypsin was then stopped by the addition of 10% CS in ECT. Cell suspensions were centrifuged at 1,500 RPM for 10 minutes and aspirated to achieve a single cell suspension in the medium required for individual experiments, and diluted to the concentration desired.

Effects Of Vanadate On Cell Growth

Cells (C13, Py and ASV) were grown to confluence, trypsinised and resuspended in ECT. Aliquots of 0.1million cells were then added to 35mm Petri dishes in 2ml ECT and left to attach and grow for 24 hours. After this time vanadate was added at the following concentrations:-
$1X10^{-4}$ M, $5x10^{-5}$ M, $1X10^{-5}$ M and $1X10^{-6}$ M. In addition HS controls were prepared. Dishes were left for a further 1,2 or 3 days before counting.

Cells were harvested from the dishes as described above and the final cell suspensions were aspirated to achieve a single cell suspension in a total volume of 3ml. 100µl aliquots were dispensed into 20ml of Coulter Saline (0.89% NaCl, 0.01% NaN_3) the cell numbers were counted using a Coulter Counter ZB.0050, and values used to calculate the total number of cells per dish. This was repeated after 1, 2 or 3 days in culture with vanadate, and in parallel, replicate dishes were fixed with 4% formaldehyde in PBSa, and stained with 0.1% solution Kenacid Blue.

Cloning Efficiency

Cells (C13 and Py) were grown to 2/3 confluence, harvested and resuspended in Hams F10 at concentrations of 1000, 500 and 250 cells per ml. 0.5ml of these concentrations were then added to replicate 9cm Petri dishes in a total volume of 10ml Hams F10 and the following treatments applied:-
$1X10^{-4}$M, $1X10^{-5}$ M, $1X10^{-6}$M vanadate and equivalent amounts of HS as control.

C13 cells were grown to 2/3 confluence and incubated in $1X10^{-4}$ M vanadate for 0,2,6 and 18 hours. After this time the monolayers were harvested and resuspended in Hams F10 as described above. 500, 250 and 125 cells were then added to replicate 9cm Petri dishes in a total volume of 10ml Hams F10.

After 10 days growth cells were washed with PBSa, fixed with 4% formaldehyde in PBSa for 15 mins, washed with water and stained with 0.1% Kenacid blue for 10 minutes, washed with water and allowed to dry. Colonies were then counted and plating efficiencies were calculated (total number colonies/ total

number cells plated x 100).

Cell Spreading/Rounding

Cells (C13,Py and ASV) were used at 2/3 confluence, harvested and resuspended at a concentration of 0.5 million per ml in HH.

13mm diameter clean glass coverslips were prepared by the addition of 200μl of 25μg/ml Fibronectin in HH, to each coverslip. These were left for 30 minutes in a moist environment at 37°C, and then washed three times with HH.

0.05 million cells were added to each coverslip, in a total volume of 1ml HH. Various additions were made, depending on the experiment:-

Vanadate - $1X10^{-4}$ M, $5x10^{-5}$M, $1X10^{-5}$ M and $1X10^{-6}$ M.

Colchicine - $1X10^{-4}$ M

Arterenol - $1X10^{-3}$ M

db cAMP - $1X10^{-3}$ M

+ IBMX - $1X10^{-3}$ M
(Isobutyryl-1-Methyl Xanthine)

Ouabain - $1X10^{-3}$ M.

Cells were then incubated at 37°C and at hourly intervals the cells counted. A sweep count of fields along the maximum diameter of the coverslip was performed , to monitor cell loss, and the proportion of cells rounded or spread on each coverslip, was calculated. Cells were scored as rounded when seen solely as bright spheres in phase contrast.

Cell density experiments were carried out as described

above, the numbers of cells added per coverslip being varied between 0.1 and 0.75 million cells. Spread and unspread cells were then counted separately after the first hour by which time there was maximal cell spreading. The proportions of rounded and spread cells were then calculated.

Differential trypsinisations were carried out on 2/3 confluent cultures. Cells were trypsinised for 1/4, 1/2, 1 and 4 times the duration used in the normal procedure described for cell harvesting. The cells were scored after 1 hour to allow for maximal cell spreading, as above.

Autoradiography

To investigate the succeptibility of cells to vanadate-induced rounding at different stages during the cell cycle C13 cells were prelabelled with ^{3}H Thymidine. Cells were incubated in ECT containing 4μCi/ml ^{3}H Thymidine for 2 hours and used immediately or left for 4 hours in medium without ^{3}H Thymidine and then used for the spreading/rounding assay described above. The cells were left on fibronectin-coated coverslips for 1 hour, and then the coverslips were washed twice with ice cold HS and fixed with 4% formaldehyde in PBSa for 15 minutes, washed twice with 5% TCA, washed twice with distilled water and twice with absolute alcohol. Once dry the coverslips were attached to slides with ClearMount, cells uppermost.

Under a red safe light the slides were dipped into a 2:1 solution of double-deionised water: Ilford Nuclear Emulsion prewarmed to 57°C. Slides were left near vertical, to dry for 20 minutes and placed in plastic slide boxes in tins with dessicant.

The tins were wrapped in foil and left at -20°C for 14 days. After this time the slides were developed with undiluted D19 and fixed with 30% Amfix. They were then washed for 30 mins with gently running water and any remaining emulsion was scraped from the back of the slides. When slides were dry they were stained with Giemsa, and when fully dried a second coverslip was mounted over the first, with ClearMount. Once this had set the number of cells spread and round and the number of labelled nuclei both spread and round was counted.

Migration Assays

C13 cells were grown to 2/3 confluence, harvested and resuspended at a concentration 0.5 million per ml ECT. 5ml of the cell suspension was placed in 25ml silicone-treated flasks and left in a gyratory shaker at 70 RPM., 37°C overnight. Aggregates were then collected and transferred to Linbro wells, approximately equal numbers of aggregates to each well. The aggregates were allowed to settle and attach for 6 hours before the following additions:-

Vanadate - $1X10^{-4}$ M, $1X10^{-5}$ M, $1X10^{-6}$ M
and HS controls.

Aggregates were left for 1, 2 or 3 days before being fixed and stained as described above.

Cell migration from the aggregate was measured by means of an eye-piece graticule with calibrated concentric circles. The largest circle over which three of more cell processess projected was taken to be the maximum limit of migration. Mean migration values for the different treatments were found.

^{32}P-Phosphate Uptake

13mm coverslips in 90mm Petri dishes were seeded with C13 cells, and grown until they had reached about 2/3 confluence. They were then placed in sectored boxes and washed twice with HS and twice with HH, to remove any non-radioactive phosphate from the medium. 1ml of HH containing $1X10^{-8}$ M ^{32}P-Phosphate was added to each coverslip, +/- $1X10^{-4}$ M vanadate. The coverslips were then incubated at 37°C and at 5 minute intervals coverslips were picked up and washed twice with ice cold HH and dropped into scintillation vials. 10ml toluene with 0.4% PPO was added to each vial and they were counted on a Packard Tri-carb 300.

Parallel experiments were run where HH was made up with non-radioactive phosphate (Na_2HPO_4) in the range $0-1X10^{-2}$ M added. Again coverslips were incubated with 1ml HH with different non-radioactive phosphate concentrations, $1X10^{-8}$ M ^{32}P-Phosphate +/- $1X10^{-4}$ M vanadate. Coverslips were incubated for 15 minutes and washed as described above.

Secretion Of ^{35}S Methionine-labelled Protein

25cm^2 flasks of C13 cells were grown to 2/3 confluence and washed twice with HS and once with HH. 0.5μCi ^{35}S Methionine in 5ml HH was added to each bottle and left at 37°C for 2 hours. The bottles were then washed thoroughly with HS, three rinses, and 5ml HECT was added to each bottle - A $1X10^{-4}$M Vanadate

B $1X10^{-5}$M Vanadate

C $1X10^{-4}$M Colchicine

D HS control

At 0 time and 30 minute intervals thereafter 50µl samples were taken from each bottle and added to a large volume (5ml) of 5% TCA. The precipitated material was collected by filtration through 0.22µm Millipore filters in a scintered glass funnel attached to a tap vacuum system. Samples from the labelling medium (HH) were also filtered as described above. The dried filters were placed in scintillation vials and 10ml Pico-Fluor added. Samples were counted in a Packard Tri-carb 300.

DNA Synthesis

Cells (C13 and Py) were grown on 13mm diameter glass coverslips, in 35mm Petri dishes until 2/3 confluent. Coverslips were then placed in sectored boxes, washed twice with HS and 0.5ml HECT containing 4µCi/ml ^{3}H Thymidine was added to each sector. At 0, 1,2,3 and 4 hours the coverslips were dropped onto GFA filters in a millipore filter assembly, washed with HS and then with 5% TCA. The filter and coverslip were transfered to scintillation vials and 0.3N NaOH was added for 30 minutes, at 37°C. 10ml Pico-Fluor 15 was added to each vial and these were then left overnight before being counted in a Packard Tri-carb 300.

The above experiment was repeated using cells grown and then incubated in HECT with 2% and 5% serum. It was also repeated using C13 cells which had been grown in HECT with 10% serum and incubated over a period of 50 hours in 2% and 5% serum.

Protein Synthesis

The experiments were performed as described for DNA synthesis but using 0.5ml ^{35}S Methionine in HECT (0.2μCi/ml) which was added to the coverslips in sectored boxes. As above C13 and Py cells were used.

^{48}V-Vanadium Uptake

^{48}V-Vanadium ($VOCl_2$) stock was supplied in 1N HCl, this was diluted to the desired activity, in HH which was brought up to pH 8 with 0.1N NaOH, using a sample of HH at pH 8 as a colour standard. After the pH had been adjusted the diluted stocks were left to stand for various lengths of time at 37°C.

^{48}V-Vanadium Uptake By Cell Monolayers

Coverslips were seeded with C13 and grown in 90mm Petri dishes until confluent. They were then either washed twice with HS and once with HH, or washed once with HS and fixed with absolute methanol, 4% formaldehyde in PBSa or 0.2% glutaraldehyde in pBSa. Live cells, fixed cells , clean glass coverslips and coverslips coated with serum were placed in sectored boxes and 1ml HH containing 0.5μCi/ml ^{48}V-Vanadium was added to each sector, at various times after the pH had been adjusted. Coverslips were removed at 15 minute intervals, washed twice with HH and placed in Gamma counter tubes.

Other experiments were carried out as described above, using vanadate 3 or more hours after the pH had been adjusted, where coverslips were incubated at 37°C and 4°C. Similarly experiments with the addition of non-radioactive vanadate , $1X10^{-4}$ M, $1X10^{-5}$ M

and $1X10^{-6}$ M, were performed with the diluted stocks 3 or more hours after the pH was adjusted, at 37°C.

Parallel experiments were run using the diluted vanadate stock at 0.25μCi/ml.

^{48}V-Vanadium Uptake By Cell Suspensions

C13 cells were harvested from roller cultures, centrifuged in conical test tubes, washed with HH, repelleted, and then resuspended in HH with 0.5μCi/ml ^{48}V-vanadate at least 3 hours after the pH was adjusted. The cells were about 30% of the total volume. Cell suspensions were then maintained at 2°C. At 10 minute intervals 0.1ml of the suspension was microcentrifuged and pellets and supernatants separated, and placed into Gamma counter tubes. Experiments were performed using live cells, cells fixed in 0.2% glutaraldehyde, and live cells in the presence of $1X10^{-4}$ M vanadate.

In another experiment two suspensions were prepared by the addition of ^{48}V -vanadate in HH. One tube was sampled at 15 minute intervals over a period of 1 hour. At the end of this time the other tube was spun so that cells and supernatant were separated. In the meantime two further suspensions were prepared. To one, fresh vanadate was added. To the cells previously exposed to vanadate,obtained as described above, fresh vanadate was then added. All three tubes were sampled at 15 minute intervals for 1 hour as described above.

All ^{48}V-Vanadium samples were counted in a Wilj Gamma counter 2001 using energy window number 4 (^{57}Co, ^{60}Co).

Cytoskeleton Extraction

C13 were grown to confluence and $1X10^{-4}$ M vanadate added for 0,2,6 or 18 hours. The $25cm^2$ flasks were then washed twice with HH, and HH + PMSF and TAME was added for 15 minutes. This was poured off and HH + PMSF and TAME and 1% Triton X-100 added for 5 minutes, followed by HS + PMSF and TAME, 0.5% Triton X-100, DNAase 1 and 10mM $MgCl_2$ for 15 minutes. The monolayers were washed twice with HH + 1.5M KCL and finally two Versene rinses. At each successive stage 5ml of the appropriate solution was added to each flask.

In some samples the monolayers detached from the substrate, and in these cases these detached cells were collected and pelleted before each new treatment. This was most noticeable in cells which had been treated with vanadate for 6 and 18 hours.

The resultant samples were collected in 1/5 sample buffer to run on SDS-PAGE (see below).

SDS Polyacrylamide Gel Electrophoresis

The method used was that of Laemmli (1970). 6%-10% polyacrylamide gradients were used. Samples were obtained as outlined above and the molecular weight markers used are described in materials.

Immunofluorescence

C13 cells were prepared and seeded onto 13mm diameter coverslips coated with FN, as described for the cell rounding/spreading assay described on page 41. The treatments

outlined below were performed in order to obtain results from the immunofluorescence that could be directly related to the cell spreading assays described above. In view of this the following treatments were performed :-

HS	2hrs
$1X10^{-4}$ M vanadate	2hrs
$1X10^{-4}$ M colchicine	2hrs
HS	4hrs
$1X10^{-4}$ M vanadate	4hrs
$1X10^{-4}$ M colchicine	4hrs
HS/$1X10^{-4}$ M vanadate	4hrs
HH/$1X10^{-4}$ M colchicine	4hrs
$1X10^{-4}$ M vanadate/HS	4hrs
$1X10^{-4}$ M colchicine/HH	4hrs

/ indicates successive treatments

Coverslips were washed after 2 hours and treated as indicated in the last four 4 hour treatments before being processed for immunofluorescence.

All coverslips were rinsed with PBSa and fixed for 10minutes in 4% formaldehyde in PBSa, followed by two rinses in PBSa. Cells were permeabilised by immersion in acetone at -20°C for 5 minutes for the anti-vimentin antibody and for the Rhodamine-Phalloidin, or by 50% and 90% methanol each for 15 minutes at room temperature in the case of the anti-tubulin antibody. The coverslips were then rinsed with PBSa and 30μl/coverslip of the anti-tubulin antibody and 50μl/coverslip of the anti-vimentin andtbody and Rhodamine-Phalloidin added. Coverslips were left at 37°C for 30 minutes in a humid environment. Coverslips were then rinsed twice with PBSa for 10 minutes each rinse. Rhodamine-

Phalloidin treated coverslips were then mounted in 50% glycerol. The remaining coverslips were incubated at 37°C for 30 minutes in a humid environment with 50µl/coverslip of a sheep anti-mouse immunoglobulin conjugated with a Texas red fluorochrome. Coverslips were then washed twice with PBSa for 10 minutes each rinse and mounted in 50% glycerol.

Appropriate negative controls, omitting the first antibody, were performed. Py cells were also seeded onto FN coated coverslips, treated as above and stained with Rhodamine-Phalloidin. Slides were observed under X50 or X100 oil-immersion with a Vickers Photoplan microscope fitted with epi-illumination from a HBO 200W mercury-arc lamp using appropriate filters. Film used was XP1 400 at 800 ASA.

Staining of DNA

Cells were grown as described for immunofluorescence and the coverslips placed in sectored boxes. Each coverslip was rinsed with PBSa, and a 3:1 methanol:acetic acid solution was added for 2 minutes, removed and replaced with fresh solution for a further 15 minutes. The coverslips were washed with PBSa and 50µl of a 1 in 1000 dilution of Hoechst 33258 in PBSa added for 30 minutes, the cells being incubated in a humid environment at 37°C. Coverslips were then rinsed with PBSa and mounted in 50% glycerol, and observed as above.

Electron Microscopy

TEM

Cells were grown to 2/3 confluence and $1X10^{-4}$ M vanadate added for 0,2,6 and 18 hours. Cells were harvested, pelleted and washed three times in 0.2M phosphate buffer. The following procedures were then carried out at 4°C. Pellets were fixed in 2.5% glutaraldehyde for 1 hour, washed and left overnight in buffer, postfixed for 1 hour in OsO_4, washed with buffer and dehydrated with Analar alcohol. The last change of 100% alcohol was done at room temperature. Pellets were then incubated in propylene oxide and embedded in 1:1 Araldite resin :propylene oxide for 1 hour at room temperature, 3:1 Araldite resin : propylene oxide for 1 hour at room temperature and left overnight in neat resin. Pellets were transferred to EM moulds and fresh Araldite added. This was then cured at 60°C overnight.

Ultrathin sections were cut using glass knives with an LKB Ultramicrotome 3, and collected on copper grids mesh 300. Sections were stained for 15 minutes with the alcoholic uranyl acetate, washed with distilled water for 1 minute, stained with lead citrate for 15 minutes, immersed in 0.05N NaOH, and washed in distilled water, blot dried and observed in Phillips 301 TEM.

SEM

Cells were grown on 13mm coverslips and $1X10^{-4}$ M vanadate adde for 0,2,6 and 18 hours. Coverslips were washed with 0.2M phosphate buffer, fixed with 2.5% glutaraldehyde solution and washed with buffer at 4°C. Coverslips were then dehydrated with acetone and critical point dried from acetone with liquid CO_2, in a Polaron 3000 Critical Point Drier. Specimens were then coated

with 200Å gold using a Polaron SEM coating unit E5000 and observed in a Phillips SEM 500.

All photographic negatives from SEM were kindly provided and processed by the EM unit, Dept. Zoology, University of Glasgow, and from TEM by the Department of Dermatology, University of Glasgow.

RESULTS

Growth Inhibition by Vanadate

In view of the many inhibitory effects of vanadate on different enzymes described in vitro, its effects on cell growth were examined.

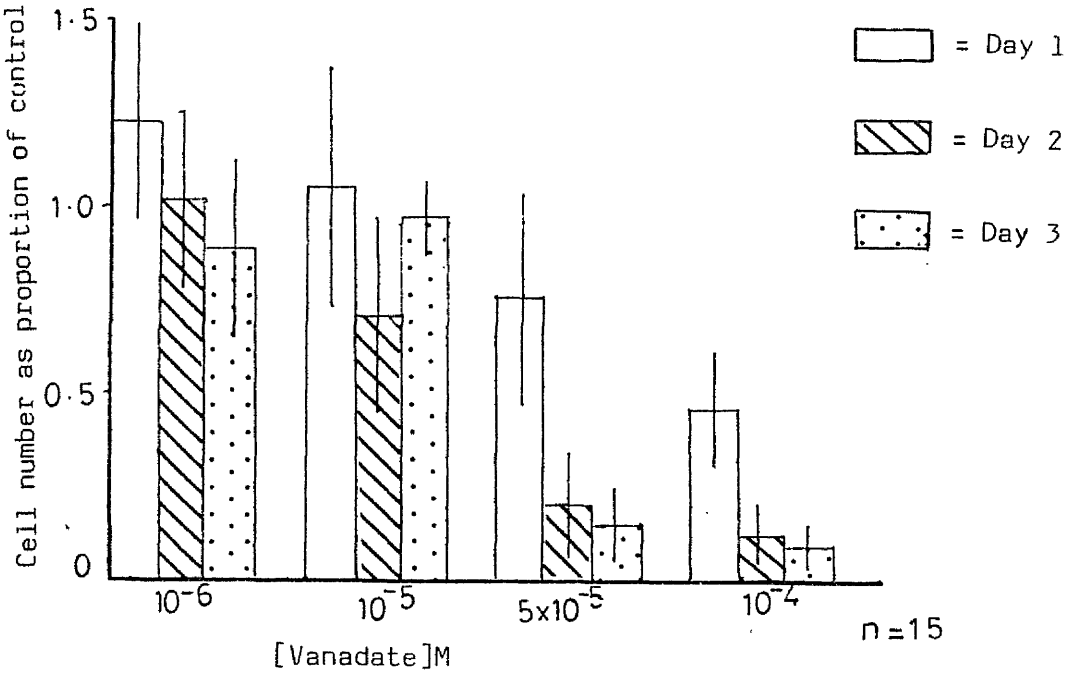
C13, Py and ASV BHK cells were incubated with $1x10^{-4}$M, $5x10^{-5}$M, $1x10^{-5}$M and $1x10^{-6}$M vanadate in ECT for 1, 2 or 3 days. In all cell lines $1x10^{-6}$M vanadate had no effect on growth. $1x10^{-5}$M vanadate had no effect on the growth of Py cells, see Figure 1b, however with C13 (Figure 1a) and ASV (Figure 1c) cells there was a significant decrease in cell number between these treatments and the controls (P=5%,and 1% respectively). With all cell types $5x10^{-5}$M and $1x10^{-4}$M vanadate were inhibitory to growth.

Thus it would appear that Py cells were less sensitive to the growth inhibitory effects of vanadate than C13 and ASV cells. See Table 4.

Physiological levels of vanadium in whole tissue have been reported to be $1x10^{-7}$M- $1x10^{-6}$M (Underwood 1977; Cantley et al 1978a). In all of the experiments described above physiological concentrations of vanadate equivalent to total tissue vanadium had no effect, either stimulatory or inhibitory, on the growth of the cells used. Concentrations of $4x10^{-5}$M vanadate over a period of 48-96 hours have been reported to be toxic to cells (Carpenter 1981). The results obtained in these experiments support this observation. However it would appear that C13 and

FIGURE 1

1a) Cl3 Cells Growth Data



1b) PY Cells Growth Data

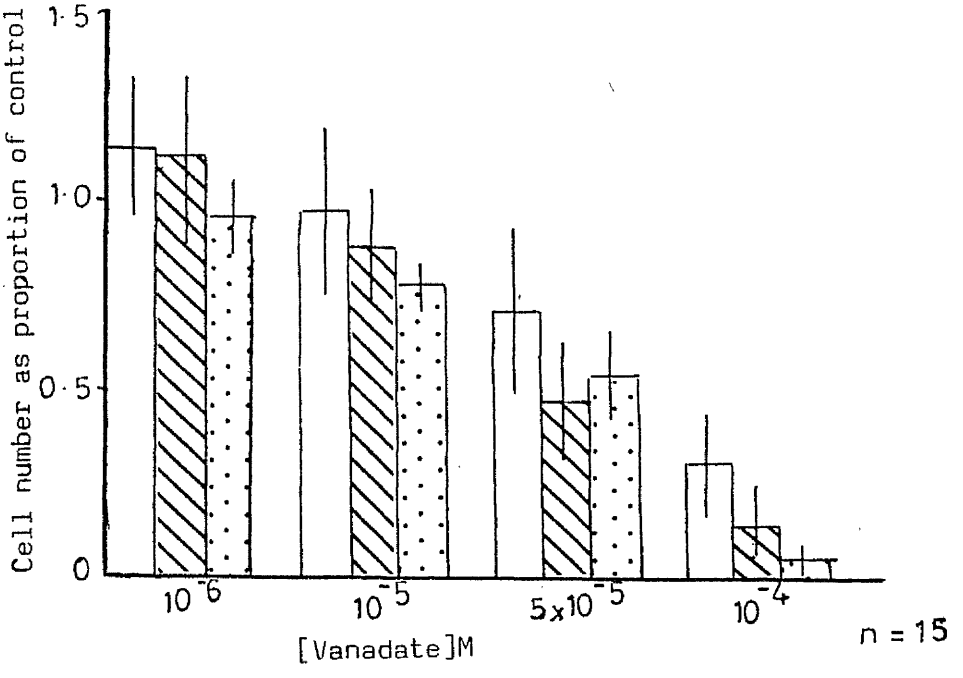
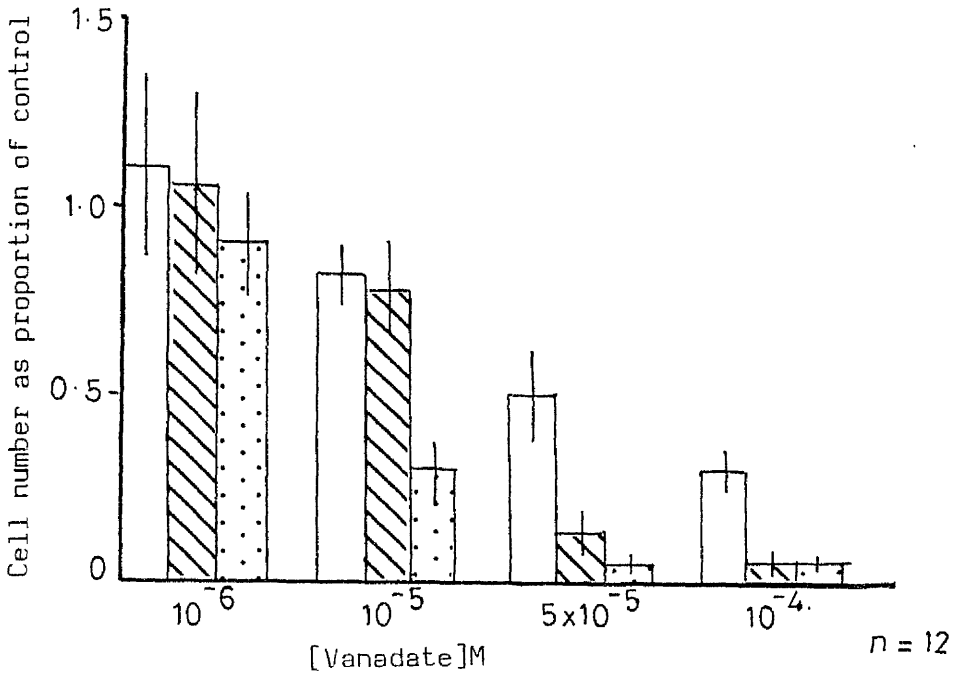


FIGURE 1

1c) ASV Cells Growth Data



Cells were grown in a range of vanadate concentrations as indicated. They were harvested from 35mm petri dishes after 1,2 or 3 days growth. Cell number was counted using a Coulter Counter and values were adjusted to give cell number as a proportion of the control (cells grown in the absence of vanadate).

Bars represent the Standard Deviations

PLATE 1

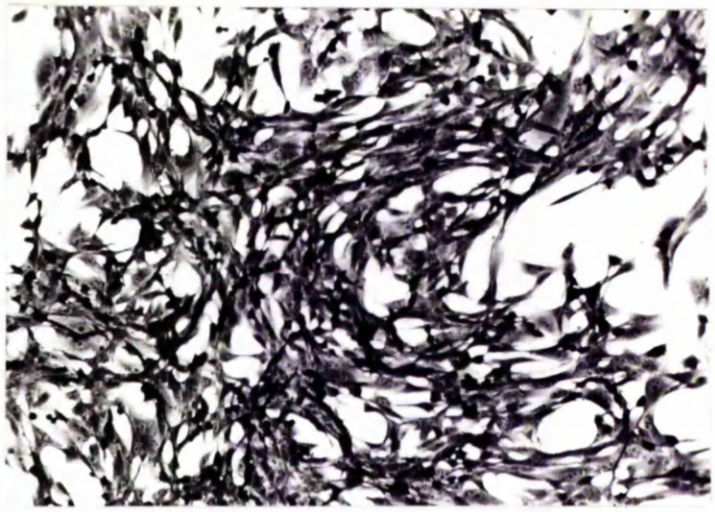
C13 Growth Experiment

a) After 2 days growth in culture C13 were well spread, forming a fairly dense monolayer.

b) After 2 days growth in the presence of 5×10^{-5} M vanadate cell number is reduced, when compared to the controls. Note many long needle-shaped cells and other partly spread cells.

Bar = 100µm

a



b

ASV cells were more sensitive to the growth inhibitory effects of vanadate, above concentrations of $1x10^{-5}M$, than the Py cells in this assay. All of the cell types described were sensitive to concentrations of $5x10^{-5}M$ vanadate and above.

Effects of Vanadate on Cloning Efficiency

Cell growth at very low cell density was then examined to see if there was any difference in sensitivity of the cells to vanadate under clonal conditions. This was considered a possibility because cells in culture condition the medium and there are undefined cross-feeding effects. At very low cell densities this is minimised, thus the medium requirements are more stringent with fewer cells (Bradshaw et al 1983).

Once fixed and stained, the colonies on each Petri dish were counted and recorded. At low density it appeared that both C13 and Py cells were unable to form colonies in the presence of $1x10^{-5}M$ and $1x10^{-4}M$ vanadate. Both cell lines showed clonal growth at $1x10^{-6}M$ vanadate and in the control plates.

As shown in table 5, $1x10^{-6}M$ vanadate reduced the plating efficiency of C13 cells by about 20% of the controls. Thus growth in this assay was slightly more sensitive than in the 3 day mass culture experiments (as discussed above). The plating efficiency of Py cells was unaffected at this concentration.

C13 cells had a high plating efficiency in the controls of the first experiment. This may have been a true reflection of the plating efficiency or could have been due to the formation of secondary colonies from cells floating free of parent colonies either in the convection currents of the medium or if the plates

TABLE 4

Results of t-tests performed on data for cell growth

(Data obtained from day 2 of the assay. See figures 1a,b and c for display of means and SDs)

Controls compared with	t	P
C13		
$1x10^{-6}$M vanadate	0.75	>50%
$1x10^{-5}$M vanadate	2.75	5%
$5x10^{-5}$M vanadate	8.72	<0.1%
$1x10^{-4}$M vanadate	9.06	<0.1%
Py		
$1x10^{-6}$M vanadate	1.96	10-25%
$1x10^{-5}$ M vanadate	1.78	10-25%
$5x10^{-5}$M vanadate	8.88	<0.1%
$1x10^{-4}$M vanadate	9.31	<0.1%
ASV		
$1x10^{-6}$M vanadate	0.76	50%
$1x10^{-5}$M vanadate	4.63	1%
$5x10^{-5}$M vanadate	8.66	<0.1%
$1x10^{-4}$M vanadate	9.25	<0.1%

C13 n = 15, Py n = 15, ASV n = 12

TABLE 5

Cloning efficiencies for C13 and Py cells, in the presence and absence of vanadate

[Vanadate]	C13 No. cells plated 500	250	125	Py No. cells plated 500	250	125
control	87±7	81±13	90±6	87±9	88±10	88±7
$1x10^{-6}$M	67±11	68±14	71±18	82±7	80±11	81±9
$1x10^{-5}$M	1±1	0	0	0	0	0
$1x10^{-4}$M	0	0	0	0	0	0

C13 N = 12

Py N = 9

$$\text{Cloning efficiency} = \frac{\text{total number of clones}}{\text{total number of cells plated}} \times 100$$

Table 6

Cloning efficiencies for C13 cells pretreated with $1x10^{-4}$ M vanadate

Pretreatment Time (hrs)	No. C13 Cells Plated 500	250	125
0	52±6	56±3	52±5
2	42±13	43±4	44±8
6	23±12	16±7	17±9
18	1	0	0

N = 9

plates were disturbed during their 10 day incubation.

Pretreating C13 cells with $1x10^{-4}$M vanadate was shown to reduce the plating efficiency of these cells following growth in the absence of the vanadate (see Table 6). A 2 hour pretreatment reduced the plating efficiency by approximately 20%, 6 hour pretreatment by approximately 50 - 70% and an 18 hour pretreatment abolished the plating efficiency completely. In this experiment cloning efficiencies in control plates were lower than the previous experiment and more typical of values obtained by other workers in this laboratory.

The growth experiments described above were performed over a long time period (3 days and 10 days for the cloning efficiencies). It was decided to look at component processes of growth in the shorter term, using radioassays to measure DNA and protein synthesis.

DNA Synthesis

In the assays for the effects on DNA synthesis, different serum concentrations were used to ascertain whether vanadate could act synergistically, or differentially, with serum factors, to stimulate DNA synthesis as reported in quiescent 3T3 cells (Smith 1983).

C13 and Py cells grown and incubated in 10% serum showed progressive uptake of ^{3}HThymidine over the 4 hours of the assay. With C13 (Figure 2a) it appeared that the two highest vanadate concentrations ($5x10^{-5}$M, $1x10^{-4}$M) could have had a slightly inhibitory effect after 2 hours. Py cells again showed this slight inhibition of uptake with the higher vanadate

FIGURE 2

Cells were grown on coverslips and incubated in HECT containing 10% serum and 4μCi/ml ^{3}HThymidine and various vanadate concentrations as indicated. At 1 hour intervals the coverslips were removed, washed with HH and 5% TCA through GFA filters in a Millipore filter assembly attached to a tap vacuum system. Coverslips and filters were then placed in scintillation vials and counted together.

○ = Control

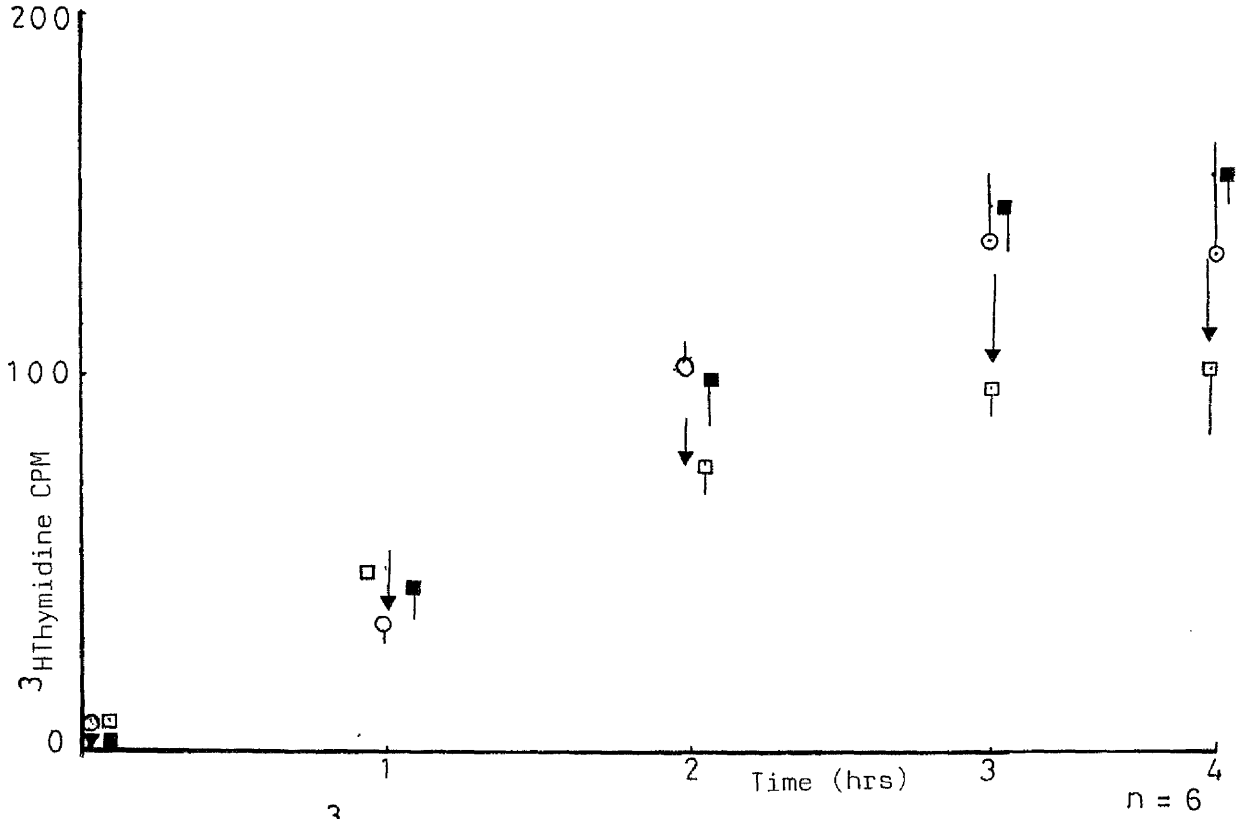
▲ = 10^{-4} M vanadate

□ = 5×10^{-5} M vanadate

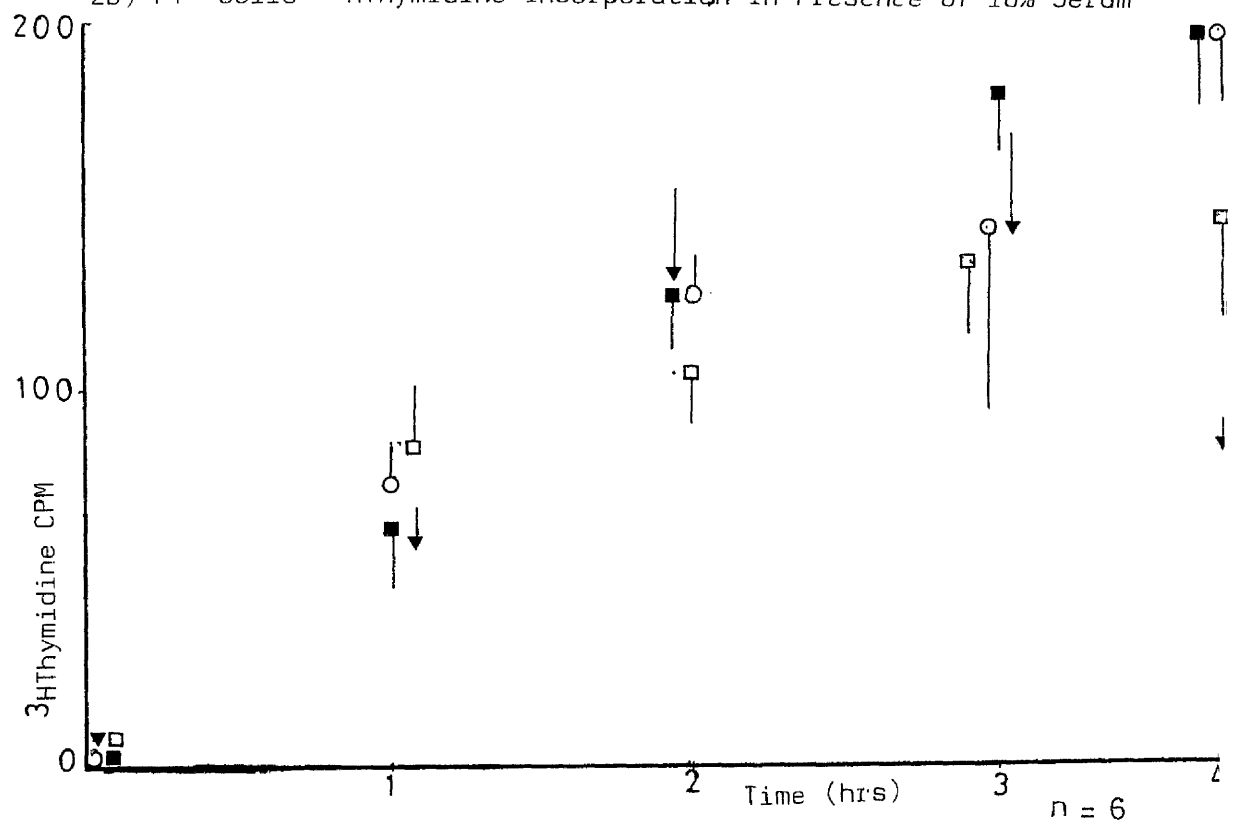
■ = 10^{-5} M vanadate

FIGURE 2

2a) C13 Cells ^{3}HThymidine Incorporation In Presence Of 10% Serum



2b) PY Cells ^{3}HThymidine Incorporation In Presence Of 10% Serum



On this and subsequent figures bars represent the Standard Deviatic
(for clarity shown to one side of the points only)

FIGURE 3

Cells were incubated in the presence of HECT containing 5% serum and 4μCi/ml ^{3}HThymidine and various vanadate concentrations as indicated. Coverslips were removed at 1 hour intervals as described previously.

● = Control

■ = 10^{-5} M vanadate

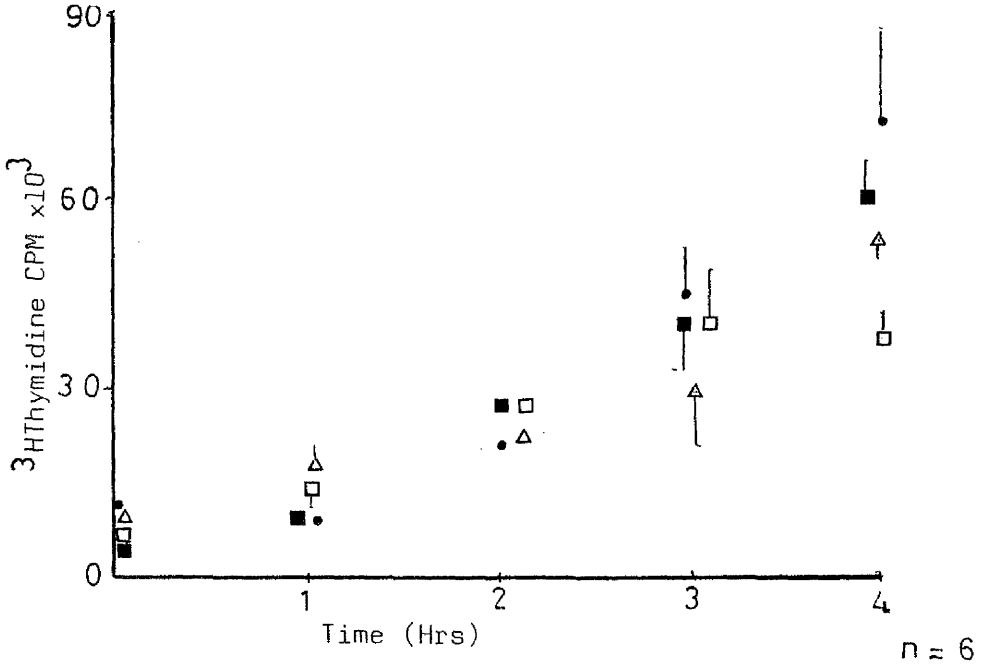
□ = $5x10^{-5}$ M vanadate

△ = $2x10^{-5}$ M vanadate

Bars represent standard deviation (shown on one side only)

FIGURE 3

3a) C13 Cells ^{3}HThymidine Incorporation In Presence Of 5% Serum



3b) PY Cells ^{3}HThymidine Incorporation In Presence Of 5% Serum

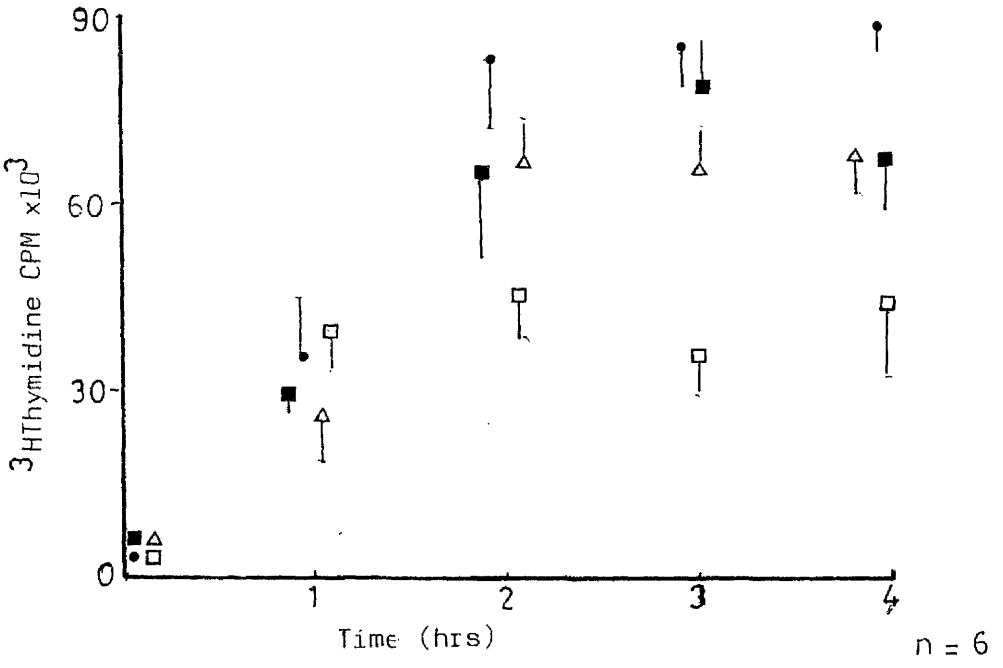


FIGURE 4

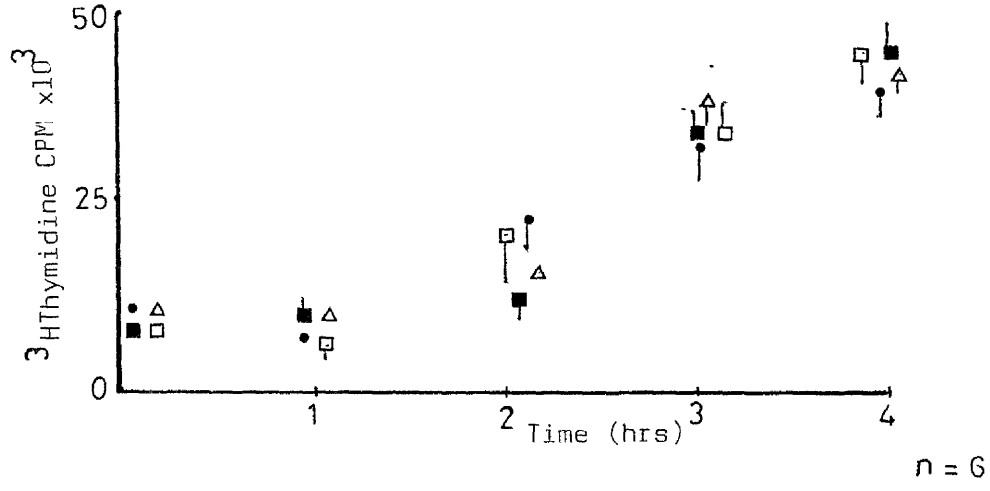
Coverslips were incubated in HECT containing 2% serum, 4μCi/ml ^{3}HThymidine and various vanadate concentrations as indicated. At intervals of 1 hour coverslips were removed and processed as described previously.

● = Control
▣ = 10^{-5} M vanadate
□ = 5×10^{-5} M vanadate
△ = 2×10^{-5} M vanadate

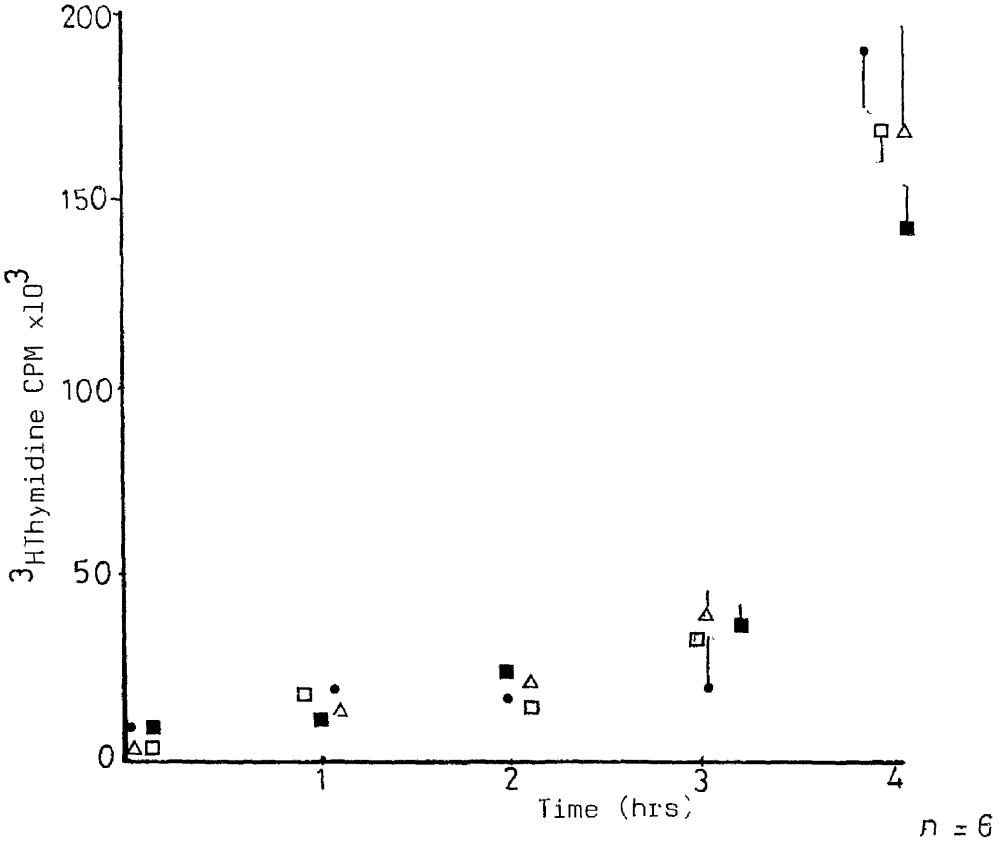
Bars represent standard deviation (shown on one side only)

FIGURE 4

4a) C13 Cells ^{3}HThymidine Incorporation In Presence Of 2% Serum



4b) PY Cells ^{3}HThymidine Incorporation In Presence Of 2% Serum



concentrations (Figure 2b), after 3 hours. The last time point for $1x10^{-4}$M vanadate is exceptionally low and it is unlikely that there was a net loss of counts from the cells. This can not simply be explained by loss of loosely attached cells as these would have been retained on the filter used. It is most probable that there were fewer cells on the coverslips used, for replicates in the experiments for this time point.

Cells that were grown and incubated in 5% serum also showed an increase in incorporation of the precursor with time (Figures 3a and 3b). There appeared to be less incorporation in those cells incubated in the presence of $1x10^{-4}$M vanadate (especially noticeable with Py cells) but no effect was seen using lower concentrations.

In cells grown and incubated in 2% serum there appeared to be a lag phase, with low levels of incorporation, followed by an increase in ^{3}HThymidine incorporation after 2 hours with C13 and 3 hours with Py cells (Figures 4a and 4b). Presumably the cells had exhausted the growth factors in 2% serum, the medium change at the beginning of the experiment serving to replenish the factors and thus allow growth.

Long term experiments were carried out over a period of 50 hours, using C13 cells, incubated in 5% and 2% serum (Figures 5 and 6). In both cases $1x10^{-5}$M vanadate caused no observable inhibition of DNA synthesis. $2x10^{-5}$M vandate produced a slight inhibition of DNA synthesis after 24 hours in 5% and 2% serum. With $5X10^{-5}$M vanadate inhibition was fully developed by 24 hours in 5% serum and 12 hours in 2% serum.

This slow onset of inhibition could explain why no great

FIGURE 5

Coverslips were incubated in HECT containing 5% serum, 4μCi/ml ^{3}HThymidine and various vanadate concentrations as indicated. At different intervals over a 50 hour period coverslips were removed and processed as described earlier.

● = Control

■ = 10^{-5} M vanadate

□ = $2x10^{-5}$ M vanadate

○ = $5x10^{-5}$ M vanadate

Bars represent standard deviation (shown on one side only)

FIGURE 5

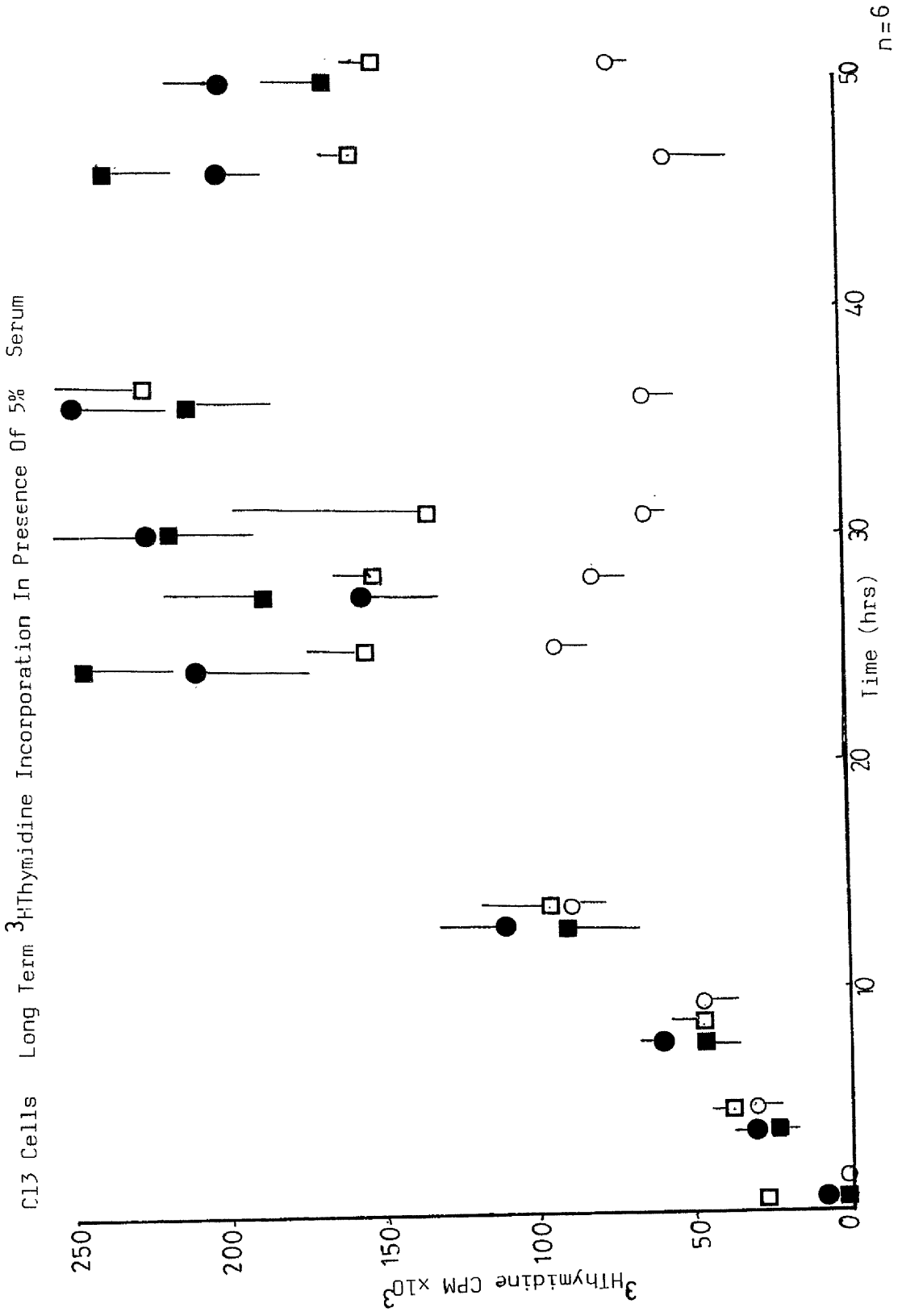

C13 Cells Long Term ^{3}HThymidine Incorporation In Presence Of 5% Serum
^{3}Hthymidine CPM x10^{3}
250
200
150
100
50
0
10
20
30
40
50
Time (hrs)
n=6

FIGURE 6

Coverslips were incubated in HECT containing 2% serum, 4μCi/ml ^{3}HThymidine and various vanadate concentrations as indicated. At different time intervals over a 50 hour period coverslips were removed and processed as described earlier.

● = Control

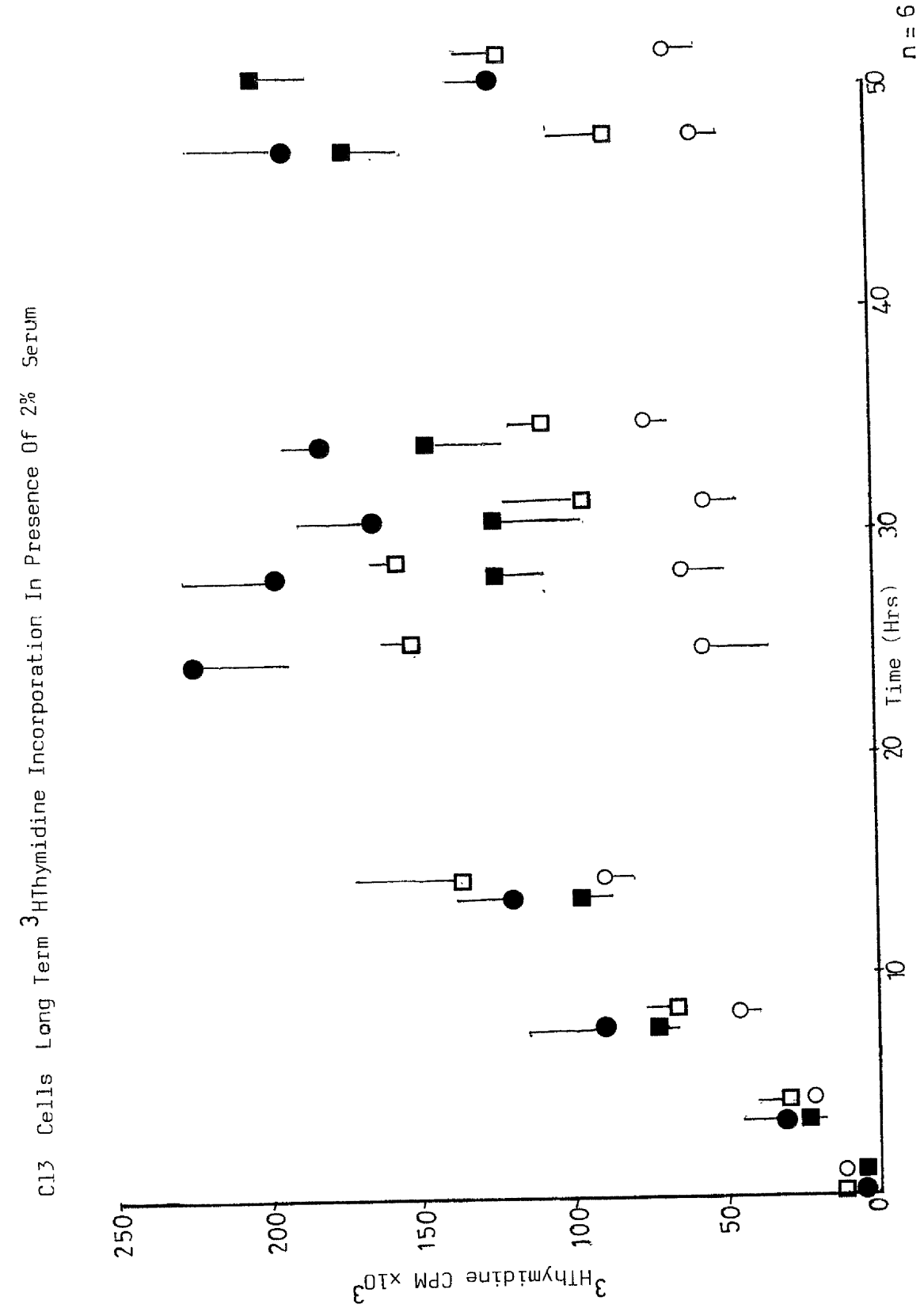
■ = 10^{-5} M vanadate

□ = $2x10^{-5}$ M vanadate

○ = $5x10^{-5}$ M vanadate

Bars represent standard deviation (shown on one side only)

FIGURE 6


C13 Cells Long Term ^{3}HThymidine Incorporation In Presence Of 2% Serum
^{3}Hthymidine CPM x10^{3}
250
200
150
100
50
0
10
20
30
40
50
Time (Hrs)
n = 6

effects on DNA synthesis were seen in the short term assays. It also indicates that over the time course of most assays performed in this work, other than the long term growth experiments, DNA synthesis was not greatly affected. Vanadate is known to inhibit DNA polymerase (Sabbioni et al 1983; and see introduction) although it has been reported to stimulate DNA synthesis in quiescent 3T3 cells (Smith 1983; and see intoduction).It would appear that the enzymes of DNA synthesis are not the primary sites of growth inhibition by vanadate.

Protein Synthesis

Coverslips were incubated in medium with ^{35}S Methionine in the presence of various concentrations of vanadate to see if ^{35}S Methionine incorporation into TCA insoluble material (a measure of protein synthesis) was affected by the vanadate. The data suggest that, with C13 cells, see Figure 7a, there was no difference in uptake of ^{35}S Methionine, with different vanadate treatments. In Py cells it would appear that there was an inhibition of protein synthesis by $1x10^{-4}$ M vanadate, incorporation of the precursor reaching a plateau after 2 hours, see Figure 7b. This was surprising in view of the generally lower sensitivity of growth of Py cells than C13 cells to vanadate. Lower vanadate concentrations also known to cause rounding of the cells (see later), had no effect on protein synthesis in either cell line. As with DNA synthesis, it would appear that the growth inhibitory effects of vanadate were not primarily due to the inhibition of protein synthesis.

FIGURE 7

Coverslips were incubated in HECT containing 0.2μCi/ml ^{35}SMethionine. Coverslips were removed at 1 hour intervals, washed with HH and 5% TCA through GFA filters in a Millipore filter assembly attached to a tap vacuum system. Filters and coverslips were placed in scintillation vials and counted together.

○ = Control

● = 10^{-4} M vanadate

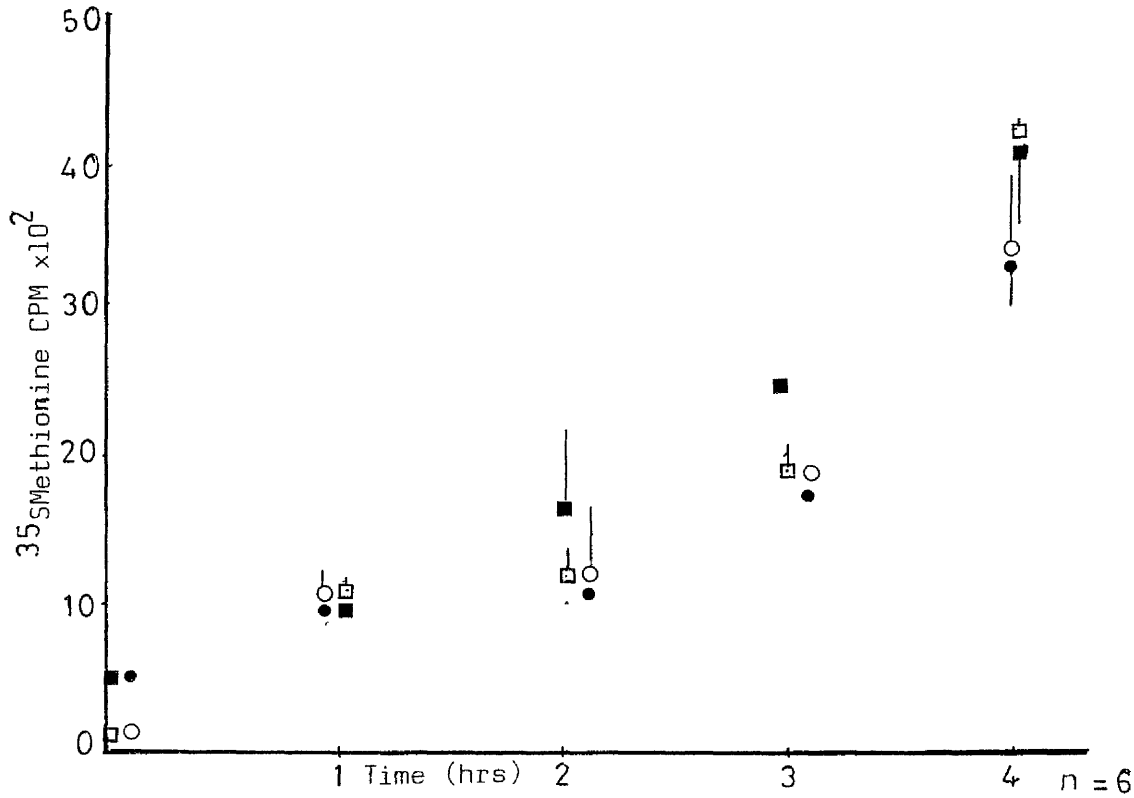
□ = 5×10^{-5} M vanadate

■ = 10^{-5} M vanadate

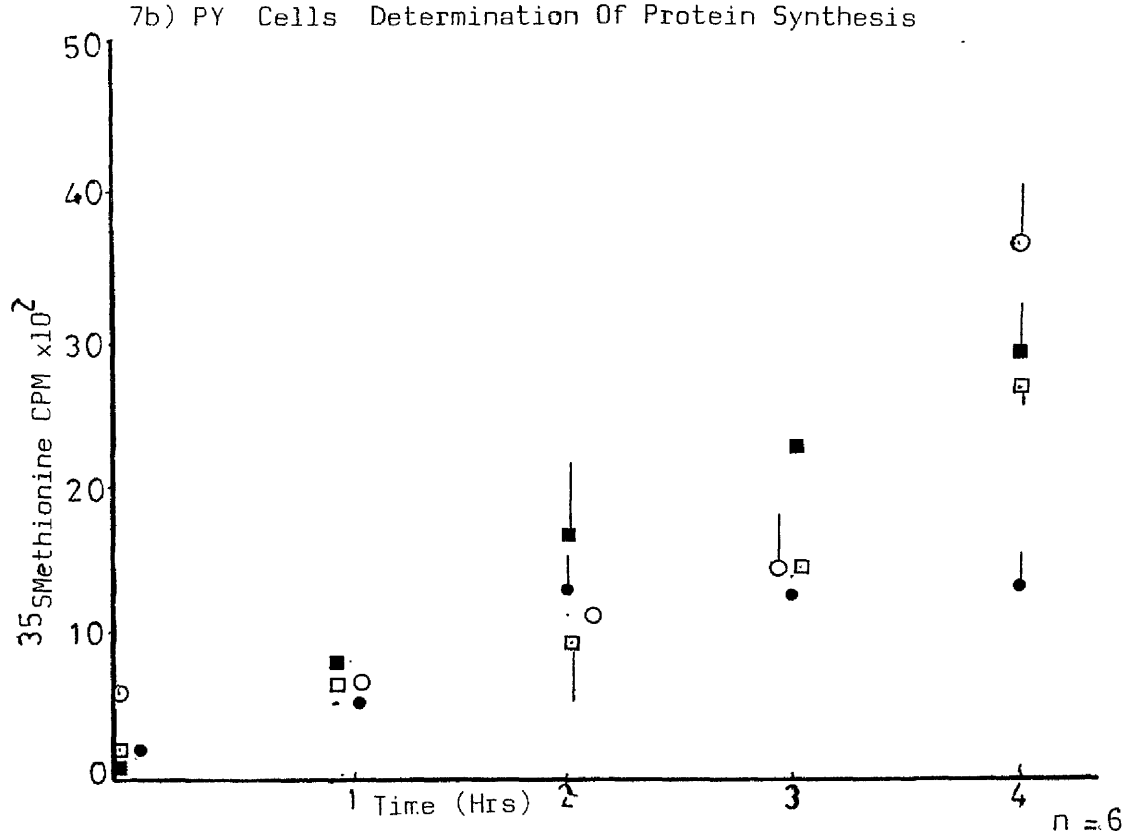
Bars represent standard deviation (shown on one side only)

FIGURE 7

7a) Cl3 Cells Determination Of Protein Synthesis



7b) PY Cells Determination Of Protein Synthesis



Migration Assays

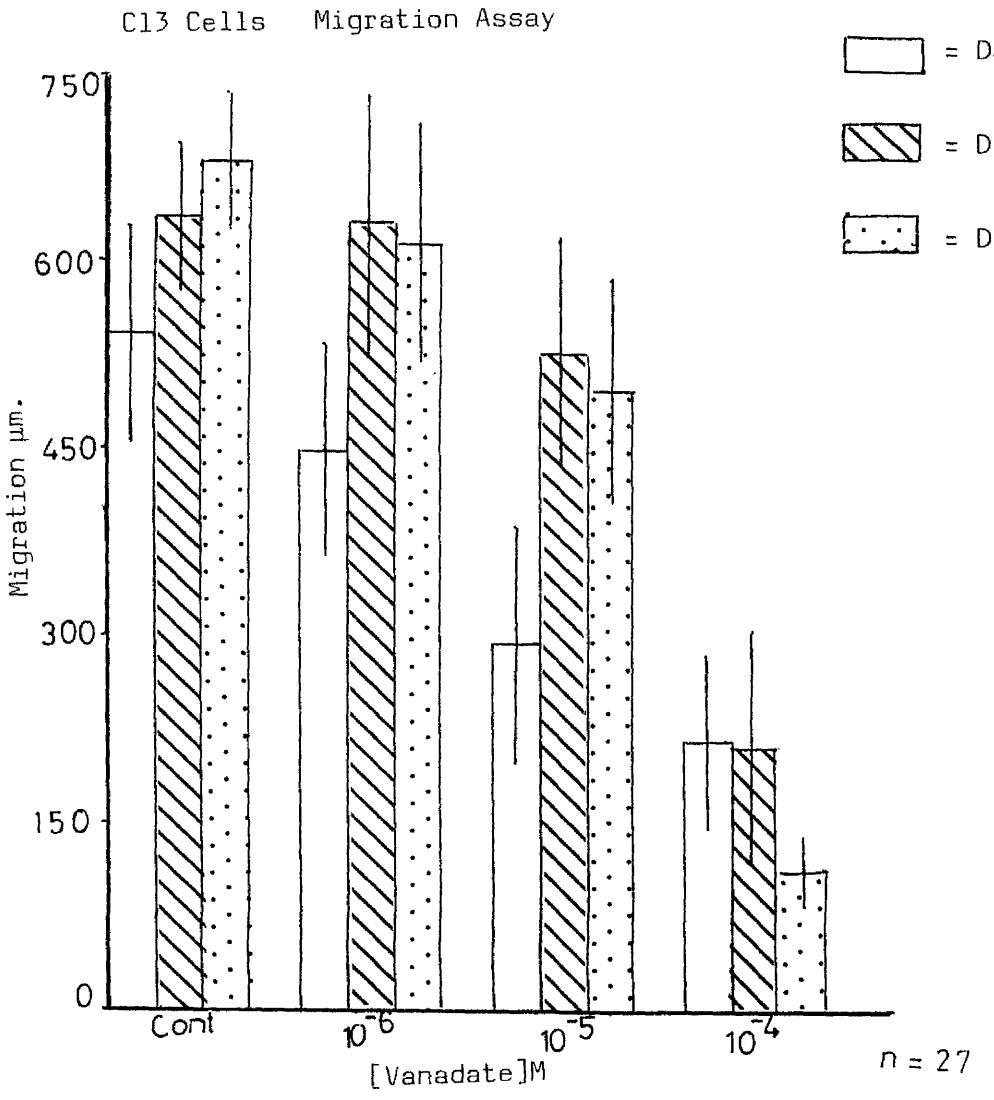
Emigration of cells from preformed aggregates was measured in the presence of various vanadate concentrations to see whether any effect on cell movement could be determined.

Cell aggregates scored in this experiment were roughly uniform in size, as determined by their diameter in the concentric circle eye-piece graticule. In controls cells migrated from the aggregates to form a monolayer over the bottom of each Linbro well, the carpet of cells increasing in density with each succesive day. $1x10^{-6}$M vanadate treated cells behaved similarly, although the cell monolayer appeared to be slightly less dense, when compared to the controls. $1x10^{-5}$M vanadate showed a considerable decrease in monolayer density in this assay, the migrating cells moving a small distance from the aggregate, but in no way forming a complete monolayer over the surface of the well. Similarly with $1x10^{-4}$M vanadate, cell migration was greatly reduced. Some aggregates produced no cell processes, and in fact some of the aggregates which had failed to spread through cell emigration were lost during the fixing and staining procedure, see Figure 8 .

It would appear that aggregates incubated with $1x10^{-4}$M and $1x10^{-5}$M vanadate for 1,2 or 3 days did show an impairment of migration, ($P=<0.1\%$ in both cases), $1x10^{-6}$M vanadate appeared to have little or no effect ($P=>50\%$).

It is very difficult to separate the effects on migration of cells and growth inhibition over the time course of this assay. To see if vanadate inhibited the locomotion of cells in the short term, cells were recorded by means of a video time-lapse recorder

FIGURE 8



Overnight aggregates of C13 cells were plated into Linbro wells ± vanadate. These were left to grow for 1,2 or 3 days. After this time the cells were fixed and stained. Outgrowth from the aggregates was measured by means of a calibrated eyepiece graticule with concentric circles. Maximum outgrowth was taken as largest circle over which 3 or more cells projected.

Bars represent the Standard Deviations

TABLE 7

Results of t-tests on data for cell migration

(See figure 8 for means and SDs of data)

Controls compared with	t	P
$1x10^{-6}$ M Vanadate	3.0	>50%
$1x10^{-5}$ M Vanadate	4.7	<0.1%
$1x10^{-4}$ M Vanadate	0.77	<0.1%

n = 27

over a period of 6 hours. The aggregates were allowed to settle on the surface of the dish for one hour. During this period cells emerged from and began to migrate outward from the aggregate. $1x10^{-4}$M vanadate was added and no further cells were observed to emigrate from the aggregates. Within one hour of the addition of vanadate the only cell movement observed was non-directional movement of processes from the cells. Within two hours of the addition of vanadate many of the cells had attained the characteristic elongated needle-like shape (see Plate 1). Thus it would appear that vanadate does inhibit the migration of C13 cells in the short term.

Affect Of Vanadate On Phosphate Transport Via The Phosphate Route

It has been proposed that vanadate enters cells via an anion exchange transport system (Cantley et al 1978a). Cantley et al (1978a) reported that the uptake of vanadate into red blood cells was competitive with phosphate. However Werdan et al (1980) using rat heart muscle cells and rat heart fibroblastoid cells reported that phosphate had no effect on vanadate uptake.

Because of the discrepancies and the use of differing cell types I used ^{32}P-Phosphate and ^{48}V-vanadate to examine uptake into cultured BHK cells.

Coverslips incubated in the presence of ^{32}P-phosphate showed a steady rise in cpm with time, not surprising in view of the cells' phosphate requirement. Incubated with $1x10^{-4}$M, $1x10^{-5}$M and $1x10^{-6}$M vanadate uptake kinetics were the same as that of

the controls (Figure 9 shows data for $1x10^{-4}$M vanadate only).

On addition of non-radioactive carrier phosphate, the ^{32}P-phosphate uptake decreased as to concentration of carrier added. This was as would be expected for the lowered specific activity of the ^{32}P-phosphate by the non-radioactive phosphate. However, $1x10^{-4}$M vanadate did not affect the uptake detectably, Figure 10.

Results have been standardised to overcome the differences in labelling due to variations in initial media values. The standard deviations in the collated data were fairly large, but individual experiments showed the same trends, and replicates within experiments showed little variation.

^{48}V-vanadium was then used to investigate uptake of vanadate, to see if specific uptake into cells could be detected.

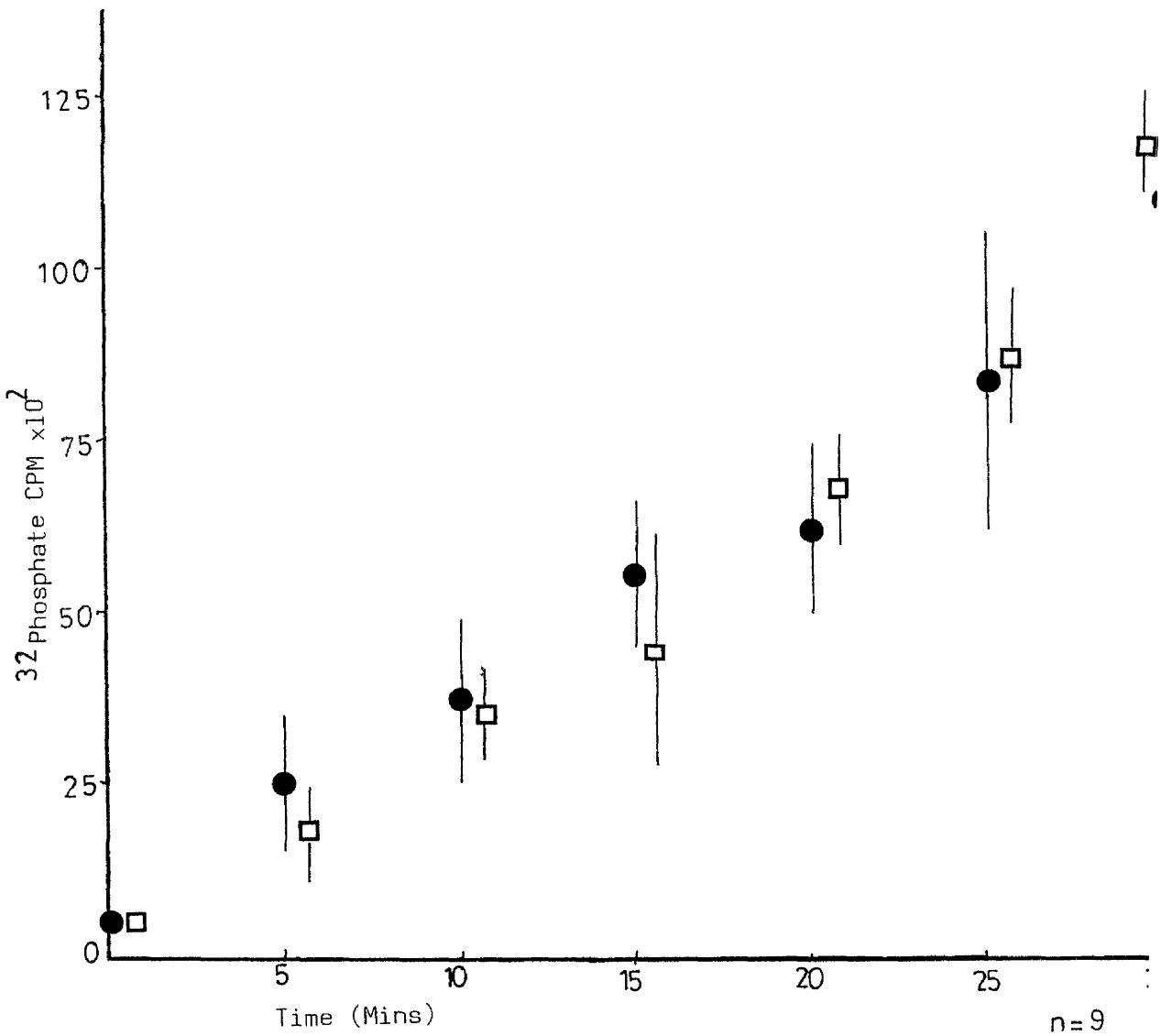
^{48}V-Vanadium Uptake

Two approaches were adopted; in the first cells grown on coverslips were incubated with radioactive vanadate; in the second cells were used in suspension where they occupied a much higher (30%) volume fraction of the medium. In the event the results obtained using the ^{48}V-vanadium were beset with problems in interpretation (see below). It would appear that there was some specific uptake, however any clear interpretation was obscured by the presence of unexplained artifacts (see below).

In particular one set of experiments with 6 replicates, results obtained with 0.5μCi/ml ^{48}V-vanadate were not reproducible with 0.25μCi/ml. Results obtained by the addition of carrier vanadate showed that the cell associated counts did not reduce in

FIGURE 9

^{32}Phosphate Uptake $\pm$ 10^{-4} M Vanadate



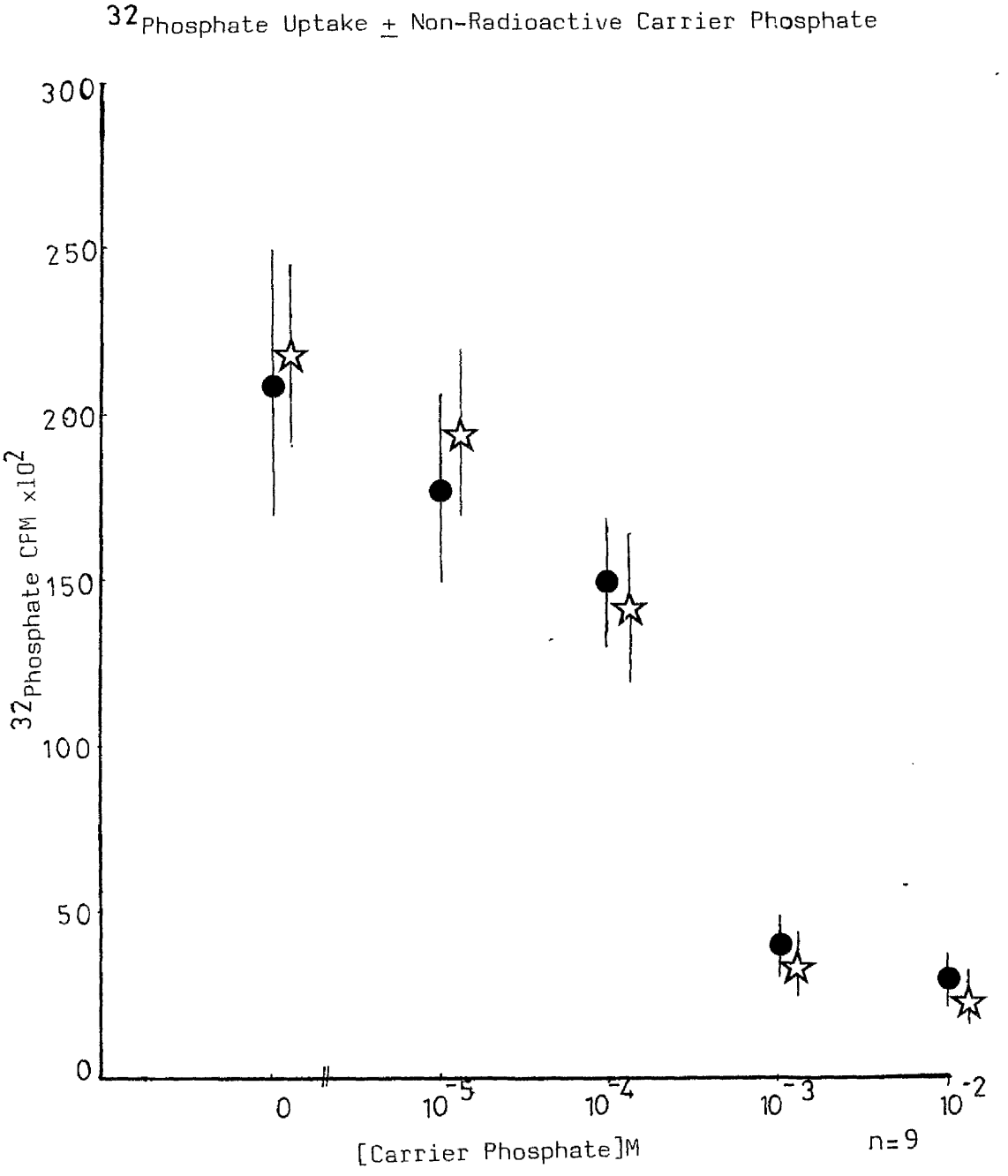
Coverslips were incubated in HH with ^{32}P-phosphate $\pm$ 10^{-4} M vanadate. At time intervals indicated, the coverslips were removed and washed twice with ice cold HH before counting.

● = Control

□ = + 10^{-4} M vanadate

Bars represent standard deviation

FIGURE 10



Coverslips were incubated for 15 minutes in a range of carrier phosphate concentrations as indicated. After this time the coverslips were washed twice in ice cold HH before counting.

● = Control

☆ = $+10^{-4}$ M vanadate

Bars represent standard deviation

proportion to the specific activity of the mixture, which suggests that some chemical species of the radioactive and carrier solutions were different.

From the uptake experiments using cell monolayers it emerged that the vanadate solution had to be left for at least 3 hours after the pH had been adjusted to pH 8. When used before this time the results obtained for ^{48}V-vanadium uptake were indistinguishable between live and fixed cells (see below). Some authors have suggested that such a period is necessary to allow the dissociation of decavanadate, which forms rapidly at pH values around 8, and then dissociates slowly (Pope and Dale 1968; Kustin and Toppin 1973).

^{48}V-vanadate Uptake Using Cell Monolayers

Using the radioactive vanadate at various times after the pH had been adjusted, at levels of 0.5μCi/ml (approximately 1×10^{-6}M), it could be seen that after 0, 1, and 2 hours at 37°C the fixed cells had values of cell-associated vanadate, equal to, or greater than the values recorded for the live cells. Similarly glass coated with serum had very high values, close to those of the live cells. Clean glass on the other hand had values about 50-80% of those for the live cells, see Figure 11a. This phenomenon was also seen at 4°C (data not shown). This is an indication that there was adsorption of vanadium onto the available surfaces, and preferential adsorption to glass.

Using vanadate that had been incubated for 3 or more hours at 37°C, after the pH had been adjusted, a different situation was observed. The live cells appeared to incorporate a much

FIGURE 11

Cells grown on coverslips were incubated in HH with 0.5μCi/ml ^{48}V-vanadium at various times after the pH had been adjusted. Coverslips were removed at 15 minute intervals washed twice with ice cold HH, and counted in a gamma counter.

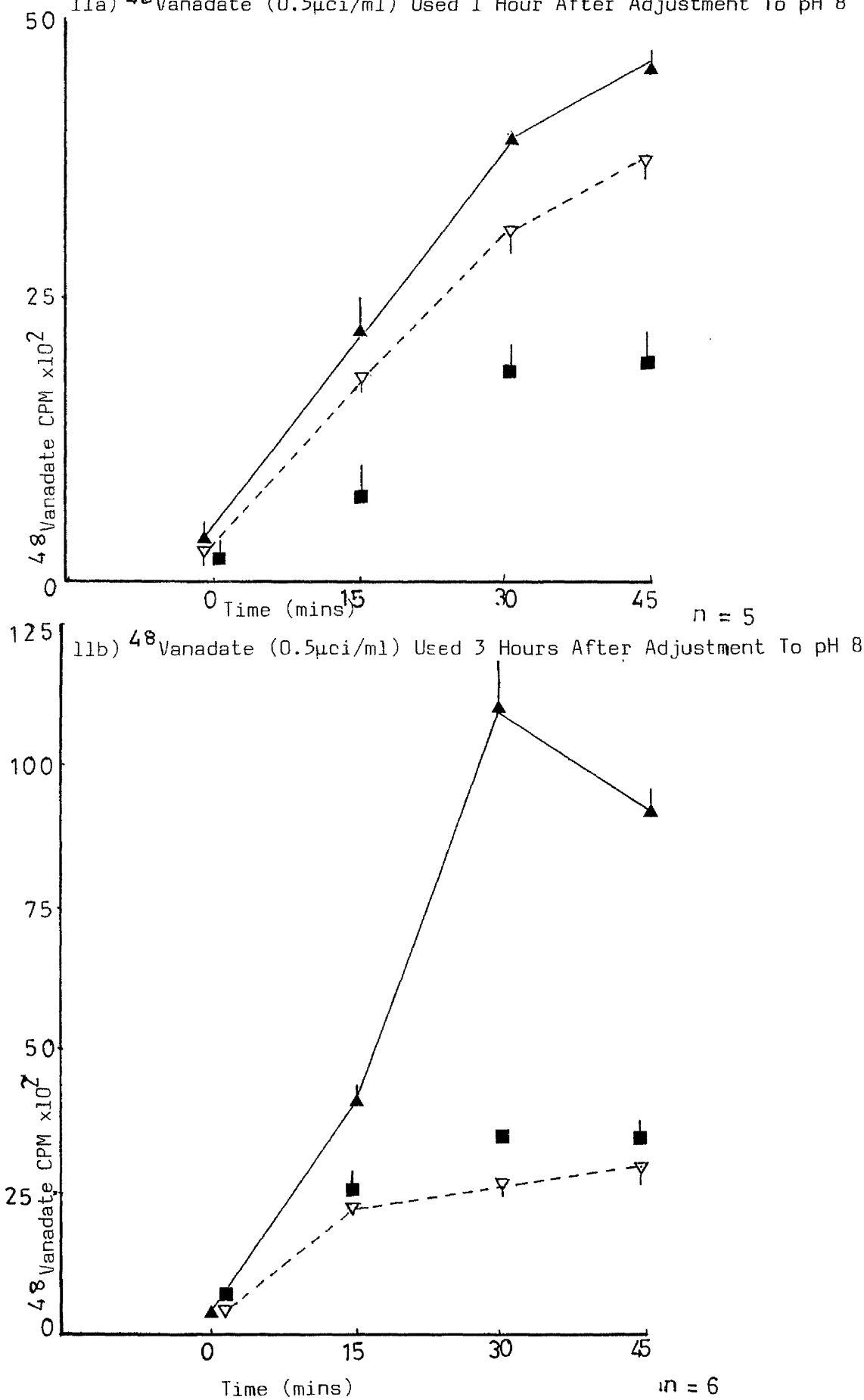
▲ = Live cells

▽ = Glutaraldehyde fixed cells

■ = Glass coated with serum

Bars represent standard deviation (shown on one side only)

FIGURE 11


11a) 48Vanadate (0.5µci/ml) Used 1 Hour After Adjustment To pH 8
50
25
0
48Vanadate CPM x10²
0
15
30
45
Time (mins)
n = 5
11b) 48Vanadate (0.5µci/ml) Used 3 Hours After Adjustment To pH 8
125
100
75
50
25
0
48Vanadate CPM x10²
0
15
30
45
Time (mins)
n = 6

larger amount of radioactivity than the fixed cells, and after a steep rise to a peak the cpm values dropped to about 80% of the maximum attained, as is shown in Figure 11b. Fixed cells showed a rise with time, however the values obtained were about 25% of the final values obtained for live cells. Clean glass and glass coated with serum showed lower cpm values than the fixed cells.

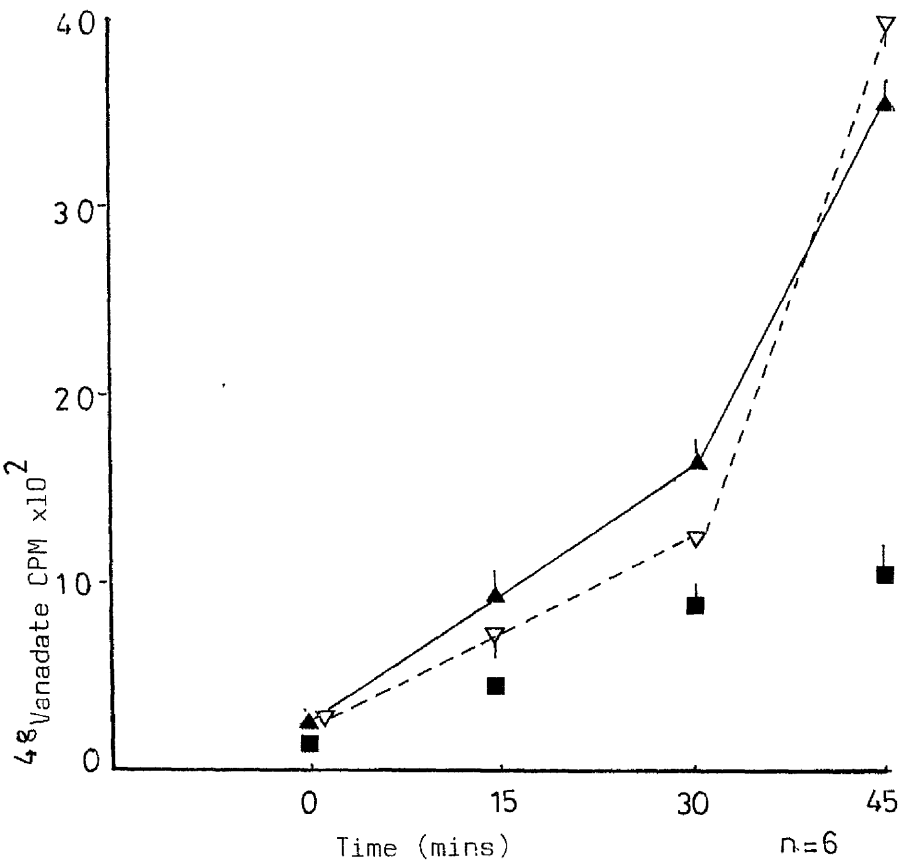
However, experiments run in parallel, using the radioactive vanadate at levels of 0.25μCi/ml, at 0, 1, 2 and 3 or more hours after the pH had been adjusted showed the uptake of radioactive vanadate to be the same over a period of 4 hours for all the above time points. Fixed and live cells gave similar values, rising throughout the time course of the assay. No peak and subsequent drop in cpm was observed. Glass coated with serum showed a similar pattern, however the average cpm values were about 30% those for live and fixed cells. Data shown in Figure 12 are from experiments conducted 3 hours after the pH had been adjusted.

Upon addition of non-radioactive vanadate, to coverslips incubated in the presence of 0.25μCi/ml, the carrier showed no effect on the association of radioactivity with the cells (Figure 13). The association of ^{48}V-vanadate with fixed cells, live cells and glass coated with serum was very similar, which means that the vanadate must bind non specifically to these proteins.

Addition of carrier vanadate to coverslips incubated in the presence of 0.5μCi/ml showed a small difference in ^{48}V-vanadate counts associated with the live cells and fixed cells. There appeared to be no effect on the glass coated with serum (Figure 14). Addition of 1×10^{-6}M vanadate caused the counts from the

FIGURE 12

^{48}Vanadate (0.25µci/ml) Used 3 Hours After Adjustment To pH 8



Coverslips were incubated in HH containing 0.25µCi/ml ^{48}V-vanadium 3 hours after the pH was adjusted. Coverslips were removed at 15 minute intervals and washed twice with ice cold HH and placed in tubes and counted in a gamma counter.

▲ = Live cells

▽ = Glutaraldehyde fixed cells

■ = Glass coated with serum

Bars represent standard deviation (shown on one side only)

radioactive vanadate to decrease to approximately half those obtained for the controls. On addition of $1x10^{-4}$M vanadate, the counts were approximately half those obtained for the controls, whereas with the addition of $1x10^{-5}$ M vanadate the counts decreased by about 20% (Figure 14). It was disturbing that decrease in ^{48}V-vanadate association with the cells on the coverslips did not seem to be proportional to the concentration of non-radioactive vanadate added.

It was calculated that the cells on the coverslip occupied approximately 0.001% of the total volume (1ml) of radioactive medium added to each coverslip. Values of V-Vanadium found were about 10 times that expected if equilibrium was to be reached across the cell membrane. Thus it would appear that there was some uptake specific to live cells, when radioactive vanadate was added at an activity of 0.5 Ci/ml, at least 3 hours after the pH had been adjusted (a total of $5x10^{-12}$ moles of vanadium per coverslip). The addition of carrier vanadate produced data that suggested that the species concerned in this uptake of ^{48}V-vanadium were not present as the major species in the stock non-radioactive vanadate.

^{48}V-Vanadate Uptake by Cell Suspensions

From the data obtained in the above experiments it would appear that there was some sort of fast initial association between a large proportion of the ^{48}V-vanadium added and cells, (Figure 15) evident in the first points of the time course 0-30 seconds.

With time the counts associated with the cell pellets declined and counts in the supernatant increased, the total counts staying the same. Addition of carrier vanadate ($1x10^{-4}$M)

FIGURE 14

Coverslips incubated in HH containing 0.5μCi/ml ^{48}V-vanadate and various concentrations of carrier vanadate as indicated. Coverslips were removed at 15 minute intervals and processed as described earlier.

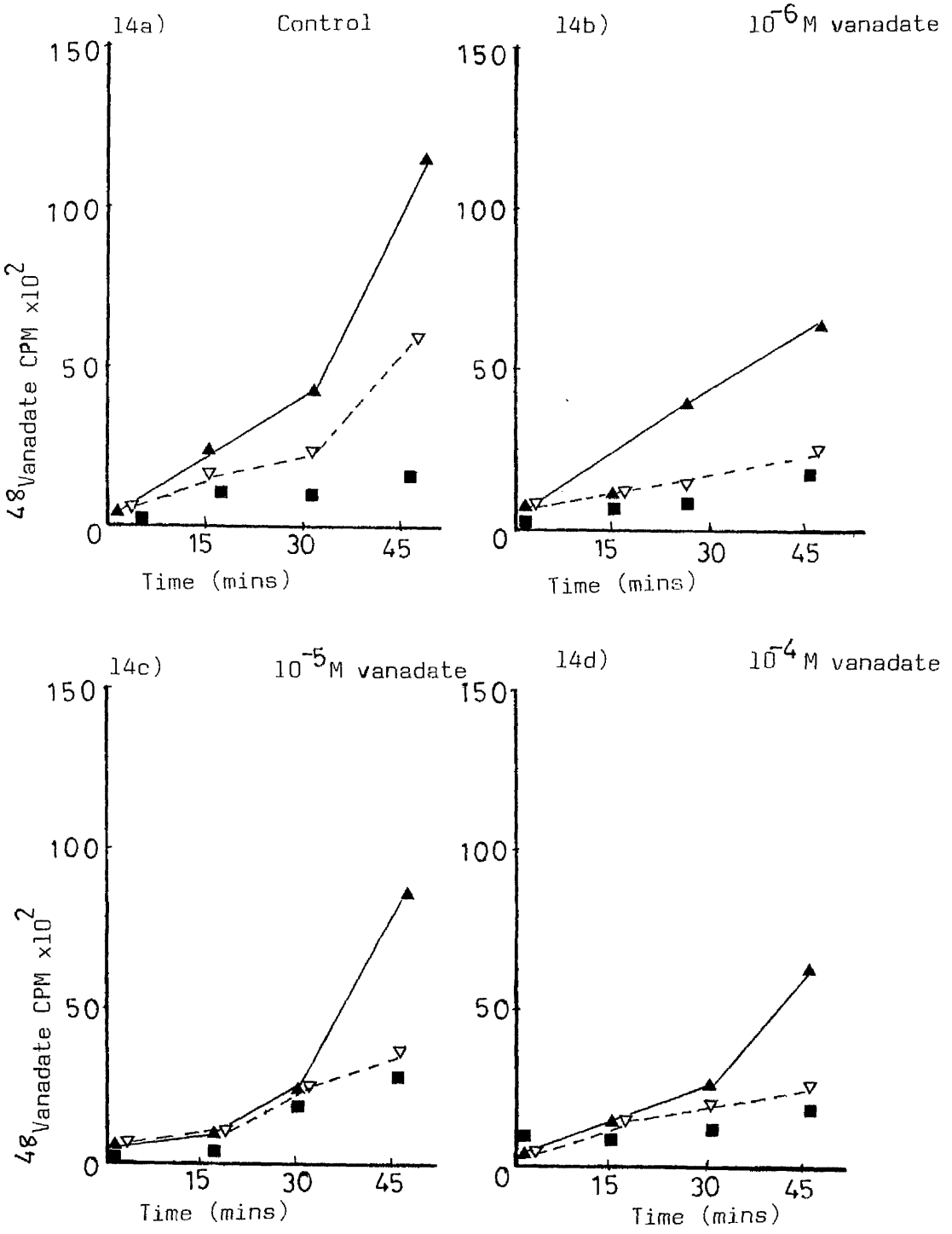
▲ = Live cells

▽ = Glutaraldehyde fixed cells

■ = Glass coated with serum

FIGURE 14

^{48}Vanadate Uptake (0.5µci/ml) ± Carrier Vanadate



n = 6

FIGURE 13

Coverslips were incubated in HH containing 0.25μCi/ml ^{48}V-vanadate and carrier vanadate at concentrations indicated. Coverslips were processed as described earlier.

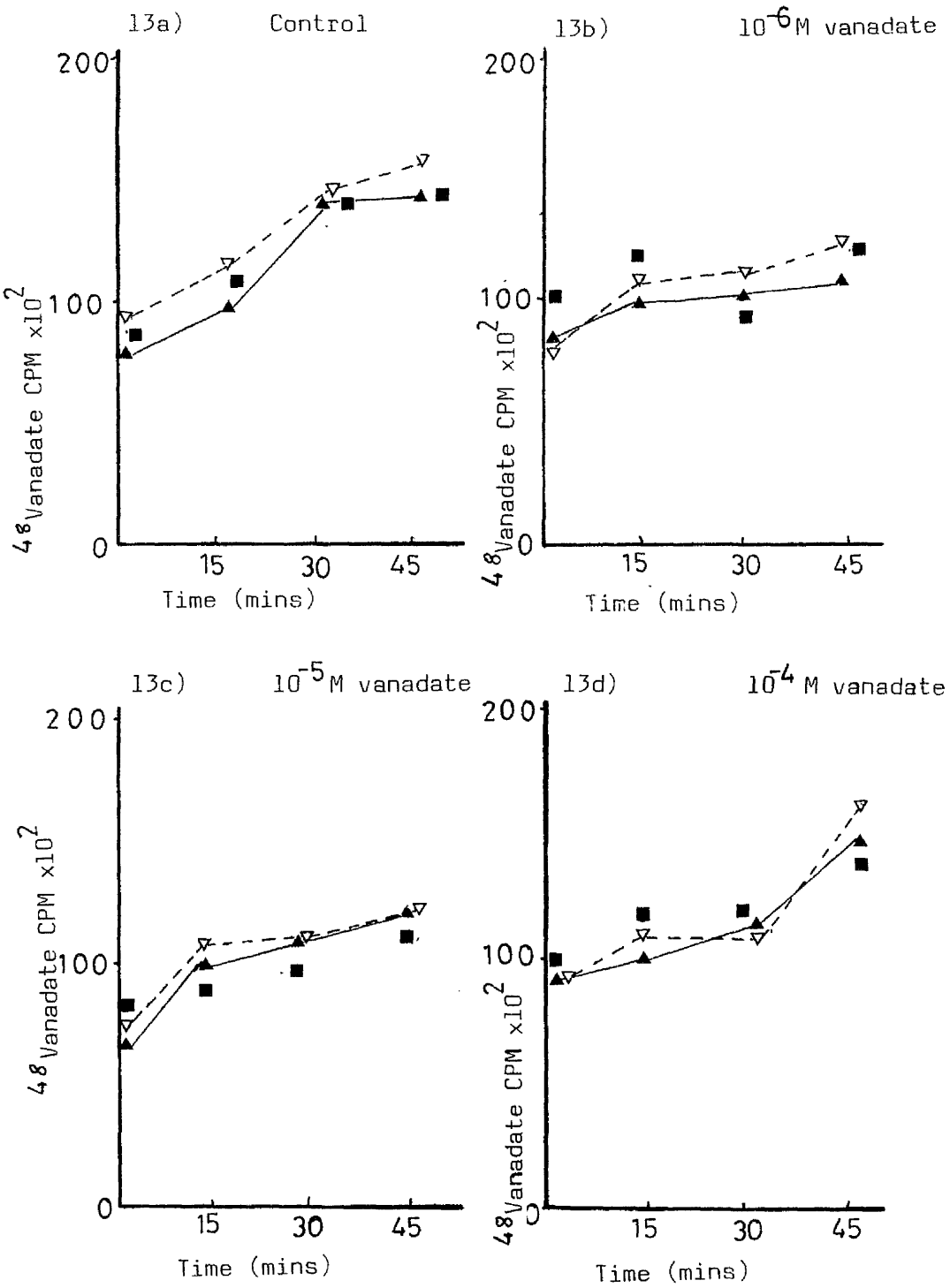
▲ = Live cells

▽ = Glutaraldehyde fixed cells

■ = Glass coated with serum

FIGURE 13

^{48}Vanadate (0.25µci/ml) Uptake ± carrier vanadate



n = 6

did not affect the distribution of radioactivity (Figure 15a). With fixed cells the initial values associated with the pellets were higher than those of the supernatant, approximately half that for the live cells, both values remained constant throughout the time course of the assay (see Figure 15b). This again suggests that the uptake of vanadium involved species not present at high concentration in the stock solution.

The fast association and subsequent release of radioactivity in these experiments suggested the possibility that the cells were modifying the chemical state of the vanadium. To test this, experiments were performed in which a vanadate solution previously incubated with cells was added to fresh cells.

In these experiments the fast association of radioactivity with the pellets was not apparent. There was a very slight decrease in counts in the supernatant and a concomitant rise in the values obtained for the pellets (Figure 16). Where fresh vanadate was added to cells already exposed to vanadate for 1 hour, radioactivity in the pellets was approximately four times greater than all the other pellet values. With time this declined to about 60% of its initial value. Supernatants showed low radioactivity initially and these rose as the pellet values dropped over the time course of the assay (see Figure 17).

It would appear that there was a fast association of ^{48}V-vanadium with live cells, even in the presence of carrier vanadate. The association with fixed cells was approximately half that for the live cells. This association was not permanent and slowly declined over a period of 1 hour. The association was not observed when vanadate already exposed to cells for 1 hour

FIGURE 15

C13 cells were incubated in suspension, in HH containing 0.5μCi/ml ^{48}V-vanadate. 0.1ml samples were withdrawn at 15 minute intervals and microcentrifuged. The pellets and supernatants were separated and counted in a gamma counter. The same protocol was followed where carrier, non-radioactive vanadate was added to the suspension (15a) or where glutaraldehyde fixed-cells were used (15b).

● = Control pellet

○ = Control supernatant

■ = + 10^{-4} M vanadate pellet

□ = + 10^{-4} M vanadate supernatant

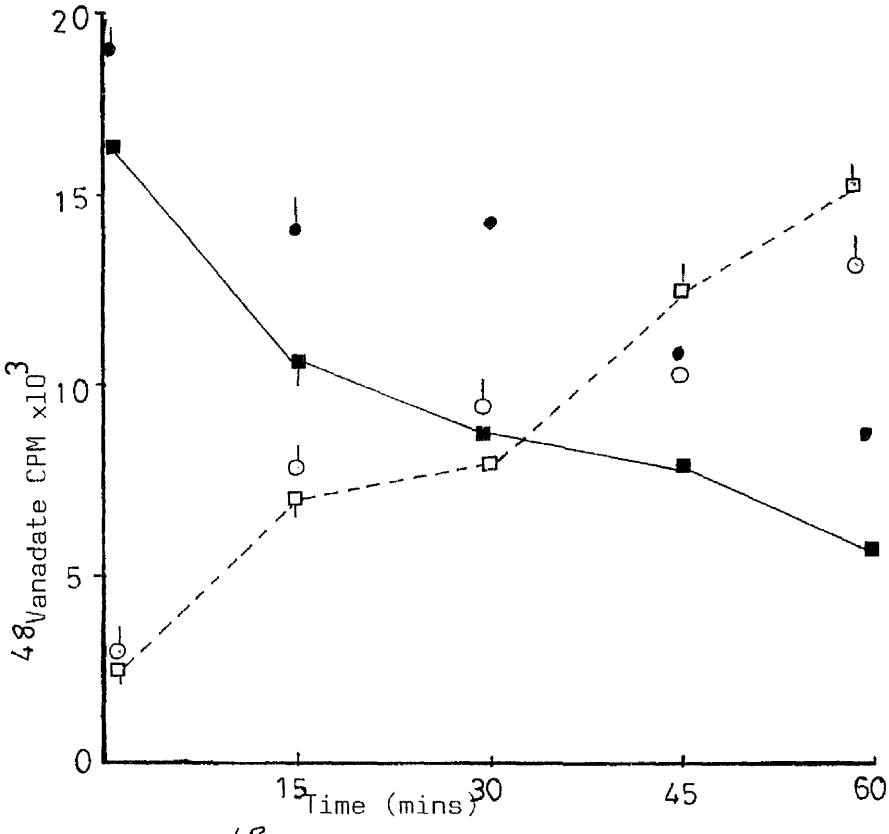
▲ = Fixed cell pellet

▽ = Fixed cell supernatant

Bars represent standard deviation (shown on one side only)

FIGURE 15

15a) ^{48}Vanadate Uptake (0.5µci/ml) Cells In Suspension ± Non-Radioactive vanadate



15b) ^{48}Vanadate Uptake (0.5µci/ml) Cells In Suspension. Live And Fixed Cells

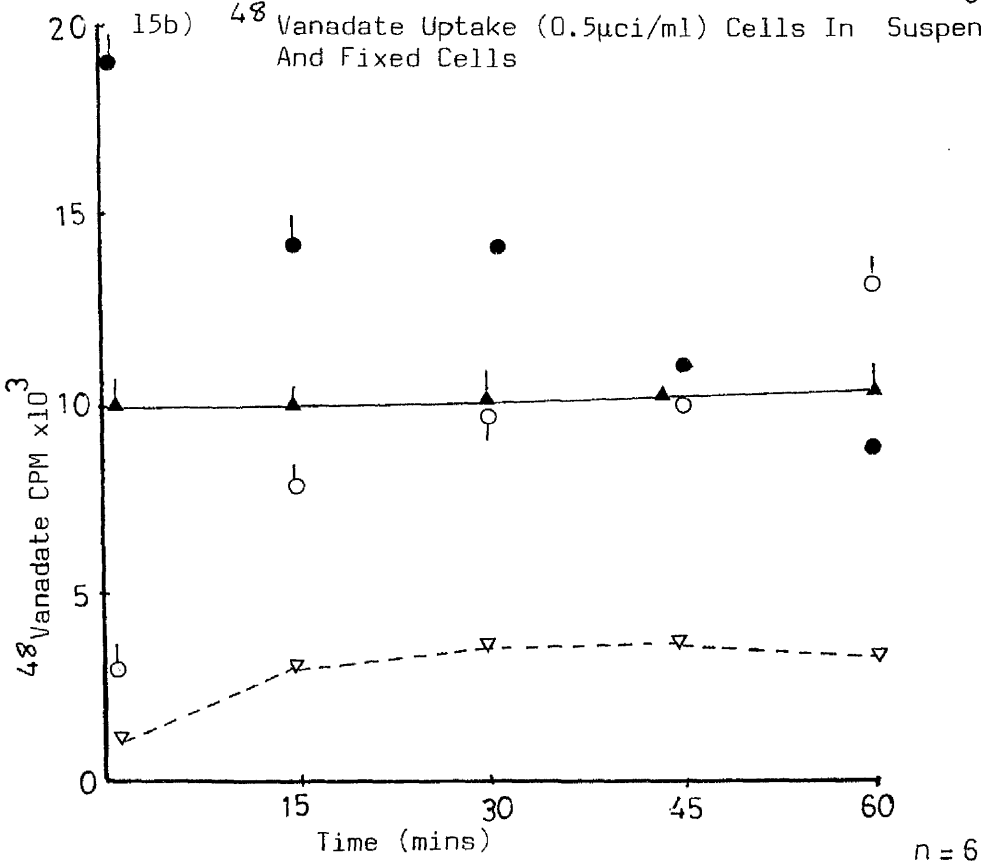
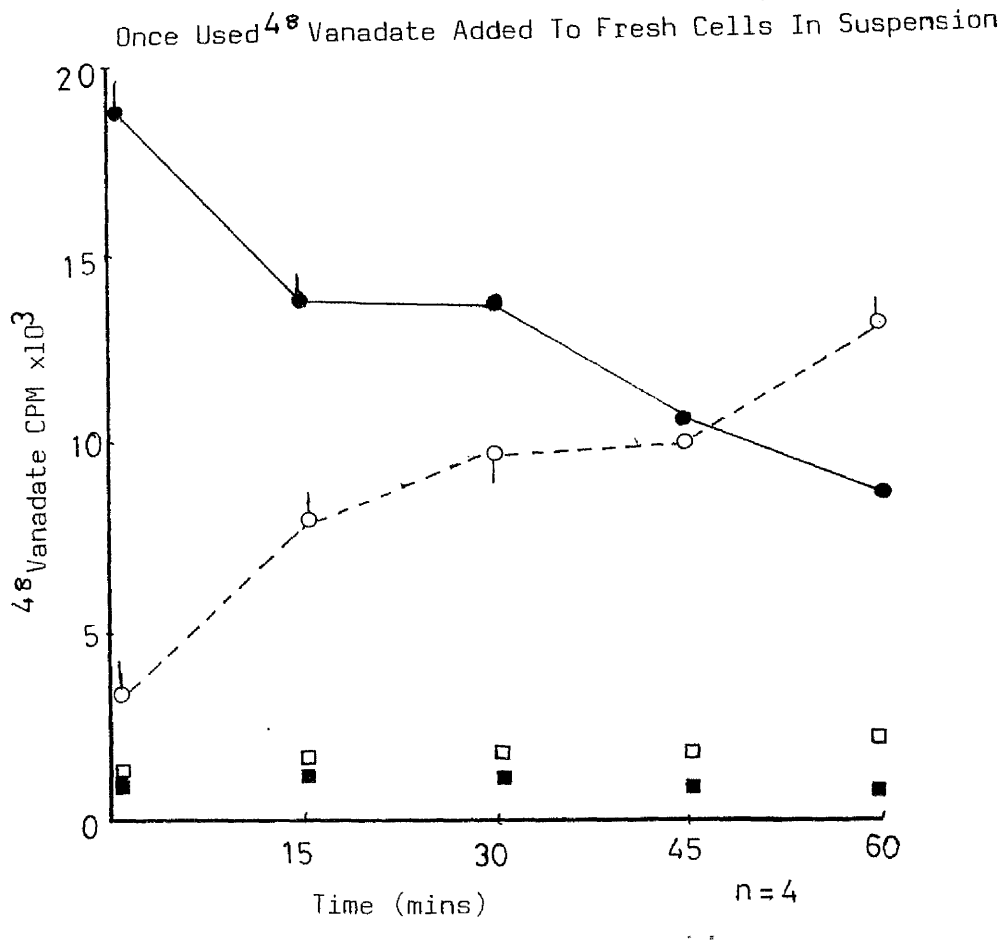


FIGURE 16



Cells in suspension in HH containing 0.5µCi/ml ^{48}V-vanadate were left for 1 hour. After this time the cells were separated from the supernatant and the vanadate that had already been incubated with cells (once used vanadate) was added to fresh cells. This was sampled at 15 minute intervals as described earlier.

● = Control pellets

○ = Control supernatants

■ = Fresh cells/ once used vanadate pellets

□ = Fresh cells/ once used vanadate supernatants

Bars represent standard deviation (shown on one side only)

FIGURE 17

A cell suspension was left for 1 hour in HH containing 0.5μCi/ml ^{48}V-vanadate. After this time the cells were separated from the supernatant (once used cells) and incubated with fresh HH containing ^{48}V-vanadate at 0.5μCi/ml. This was sampled at 15 minute intervals as described before.

● = Control pellet

○ = Control Supernatant

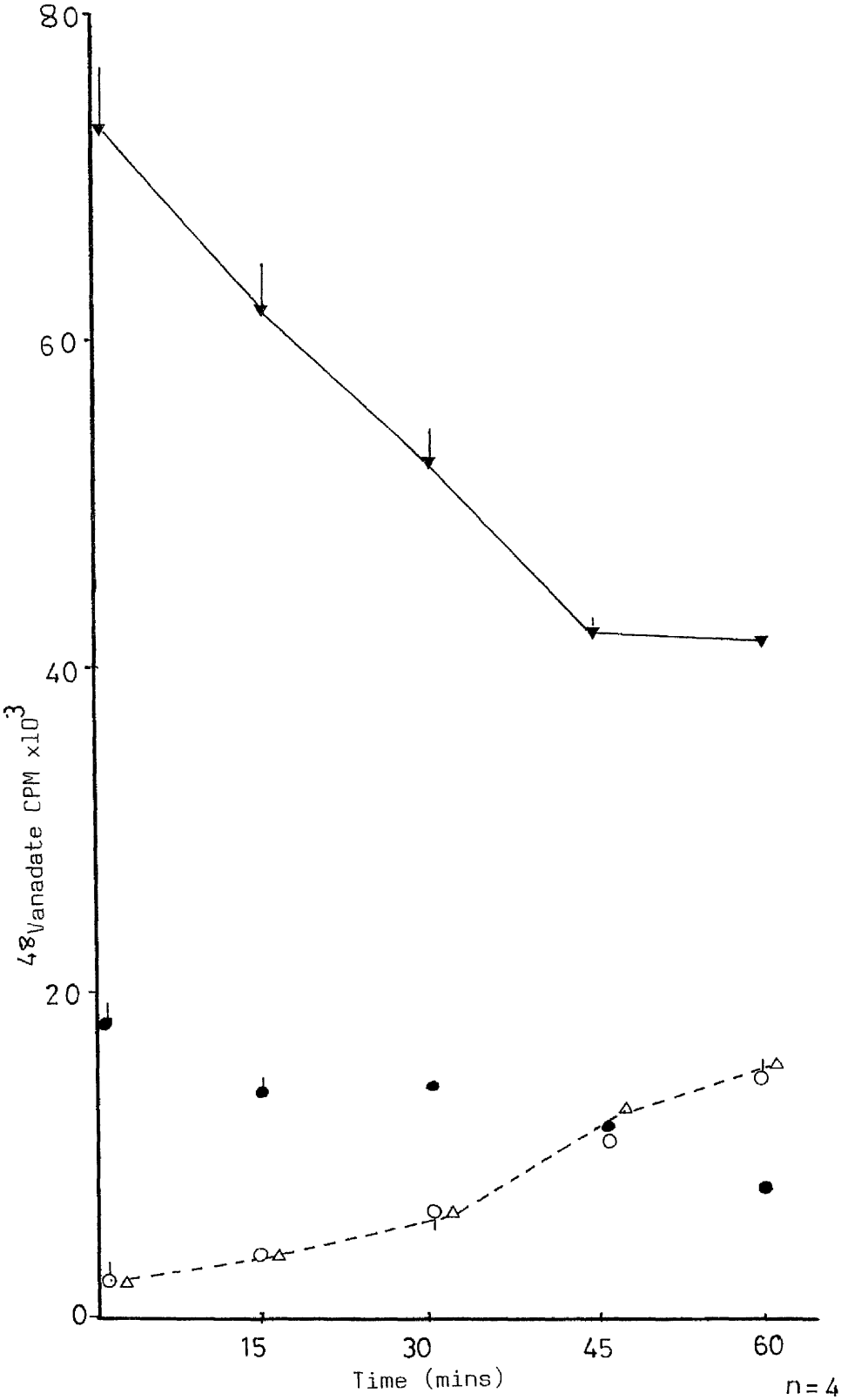
▼ = Fresh vanadate/ once used cells pellet

△ = Fresh vanadate/ once used cells supernatant

Bars represent standard deviation (shown on one side only)

FIGURE 17

Fresh ^{48}Vanadate (0.5µci/ml) Added To Cells Already Exposed To ^{48}Vanadate For 1 Hour



was incubated with fresh cells. Cells that had been exposed to vanadate for 1 hour and then incubated in the presence of fresh vanadate showed a greatly enhanced association of radioactivity.

A possible explanation of these observations is that the vanadate was chemically modified by the cells (reduced?), and released during the 1 hour incubation. It is possible that in this reduced form the vanadium was then unable to enter or bind to fresh cells.

Effects Of Vanadate On Cell Morphology: Spreading Assays

It was noticed that during the long-term growth assays described above, many of the vanadate-treated cells became rounded, over a period of several hours. Those cells that remained spread became needle-shaped (See Plate 1). In view of this shape change, it was decided to investigate the effects of vanadate on the morphology of cells seeded on fibronectin. Vanadate was found to inhibit spreading and various possible mechanisms of this inhibition were tested.

Effect Of Vandate On Cell Spreading On Fibronectin

All cell types used spread progressively throughout the time course of the assay (see Figures 18,19 & 20). When cells were incubated with $1x10^{-4}$M vanadate, C13 and Py cells showed inhibition of spreading by about 50%. i.e. about 50% of the cells remained rounded after 1 hour. ASV cells showed approximately 70% inhibition of spreading. $1x10^{-6}$M vanadate had no effect on

FIGURE 18

Cells were seeded on fibronectin-coated coverslips and incubated in HH containing various vanadate concentrations as indicated. At intervals of 1 hour the coverslips were scanned along their maximum diameter and the numbers of spread and rounded cells were counted. Results were expressed as percentage of cells spread.

For reversal experiments the coverslips were washed three times with HH after 2 hours and the cells were re-incubated with or without vanadate as indicated.

18a)

▽ = HS

▲ = 10^{-6} M vanadate

□ = 10^{-5} M vanadate

■ = 10^{-4} M vanadate

18b)

▽ = HS/10^{-4} M vanadate

▲ = 10^{-6} M vanadate/HS

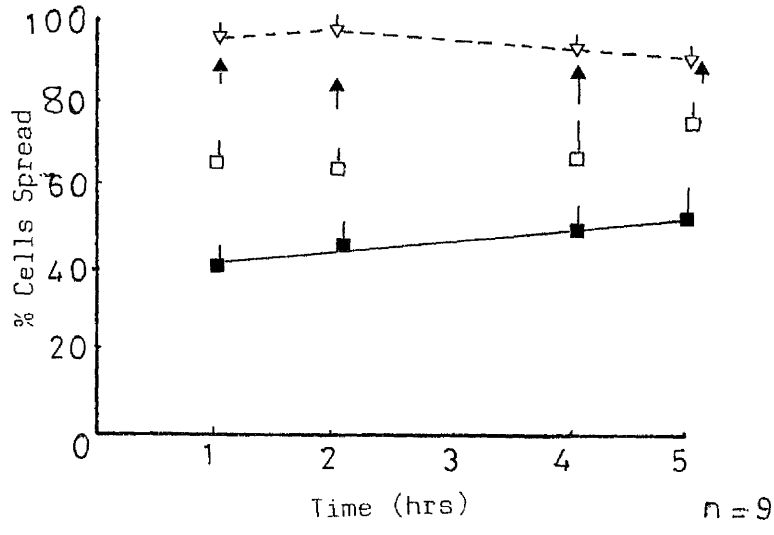
□ = 10^{-5} M vanadate/HS

■ = 10^{-4} M vanadate/HS

Bars represent standard deviation (shown on one side only)

FIGURE 18

18a) Effect Of Vanadate On Spreading Of C13 Cells



18b) Reversal Of Vanadate Effects By Washing

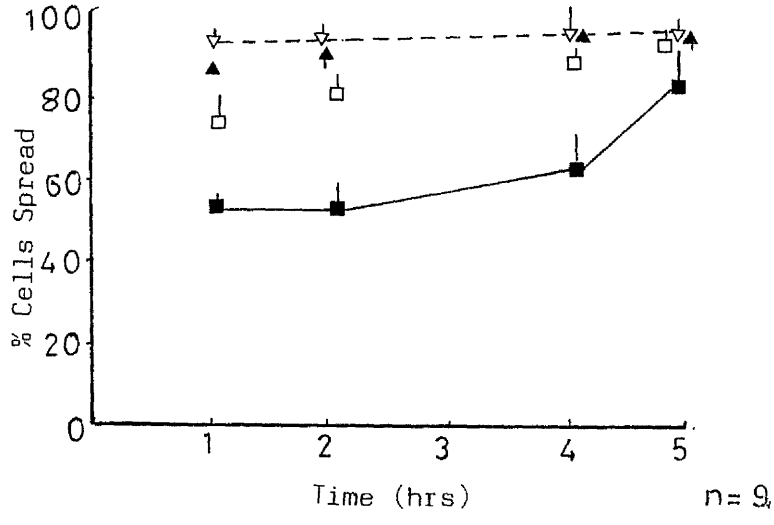


PLATE 2

Cl3 cells from spreading assay

a) Cells were well spread after incubation in HH.

b) Many cells remained rounded after incubation in HH + 10^{-4} M vanadate.

c) Addition of 10^{-4} M vanadate to cells prespread in HH had no effect on cell spreading.

d) Rounded cells began to spread after 10^{-4} M vanadate was removed by washing.

Bar = 50µm

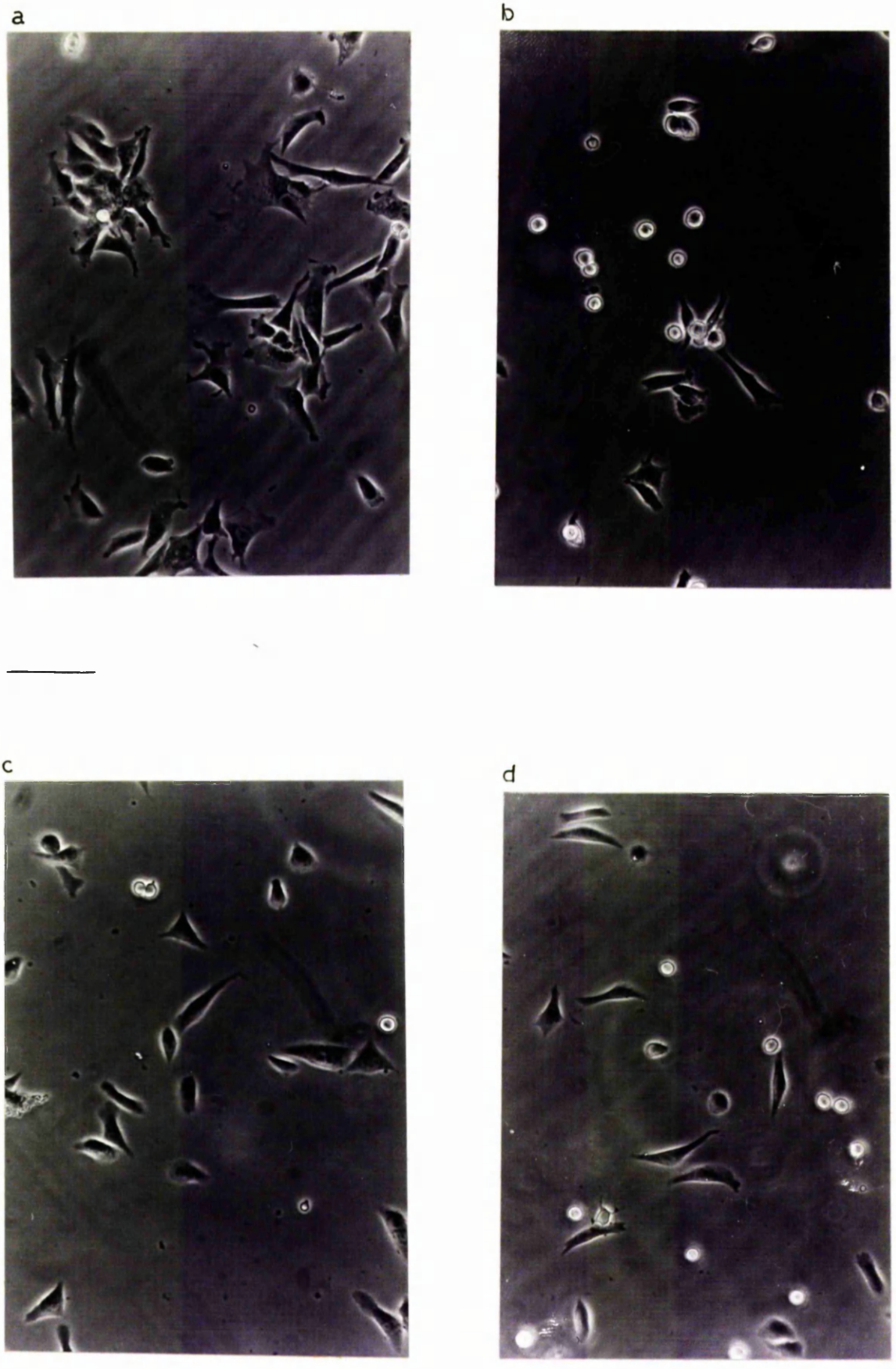
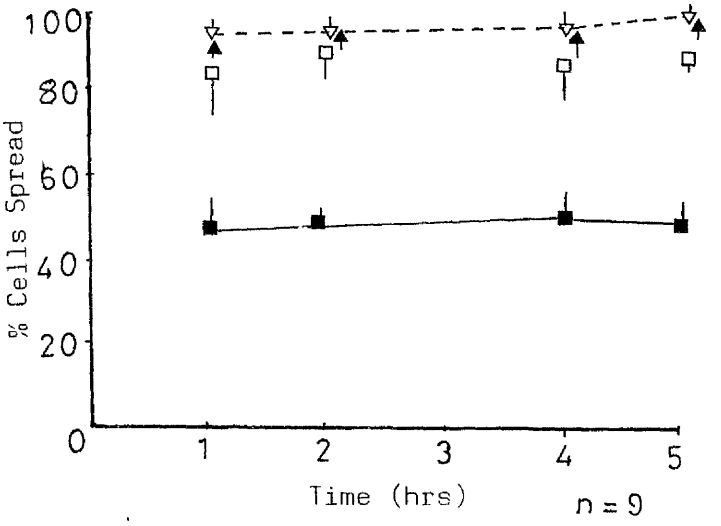
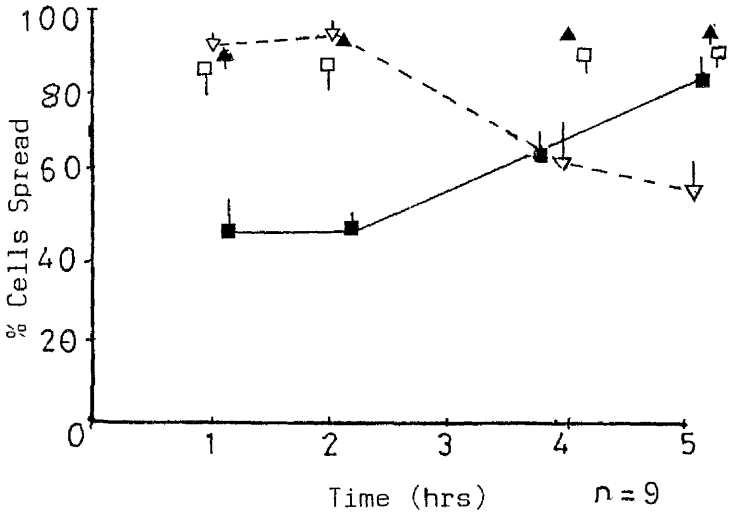

a
b
c
d

FIGURE 19

19a) Effect Of Vanadate On Spreading Of PY Cells



19b) Reversal Of Vanadate Effects By Washing



Spreading assays were performed as described previously, but PY cells were used.

19a)

▽ = HS

▲ = 10^{-6}M vanadate

□ = 10^{-5}M vanadate

■ = 10^{-4}M vanadate

19b)

▽ = HS/10^{-4}M vanadate

▲ = 10^{-6}M vanadate/HS

□ = 10^{-5} M vanadate/HS

■ = 10^{-4}M vanadate/HS

PLATE 3

PY cells from spreading assay

a) Cells incubated in HH were well spread.

b) Incubated in HH + 10^{-4} M vanadate many cells remained rounded.

c) Addition of 10^{-4} M vanadate to prespread cells caused many of these cells to round up.

d) Rounded cells began to spread after the 10^{-4} M vanadate was removed by washing.

Bar = 50μm

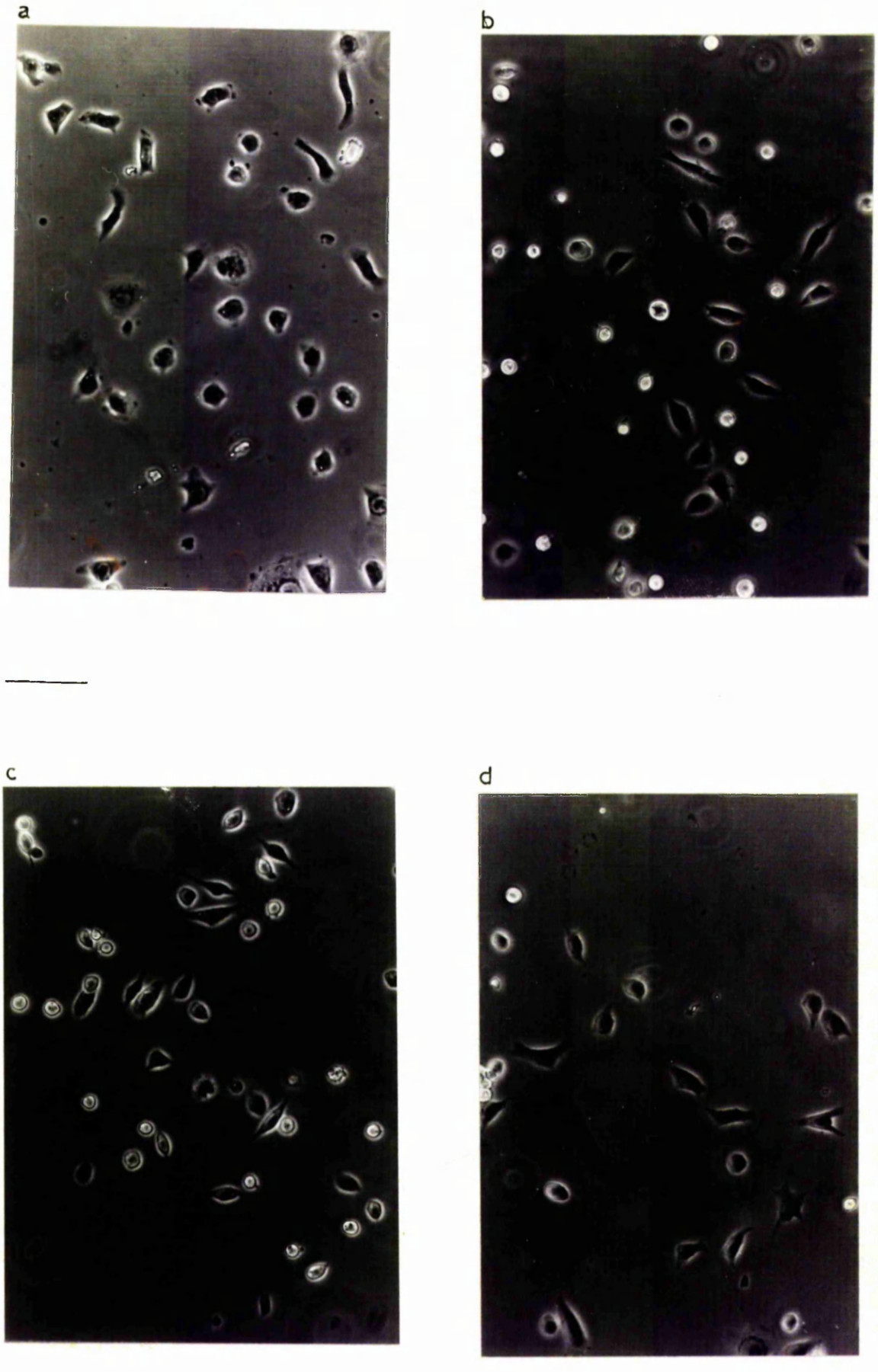
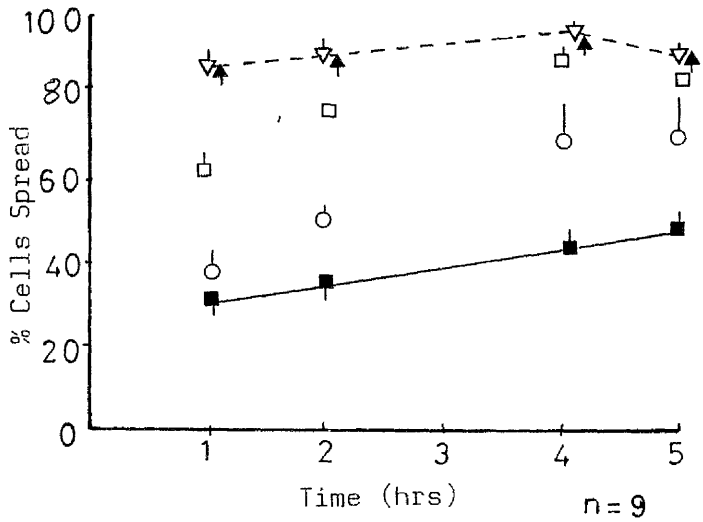
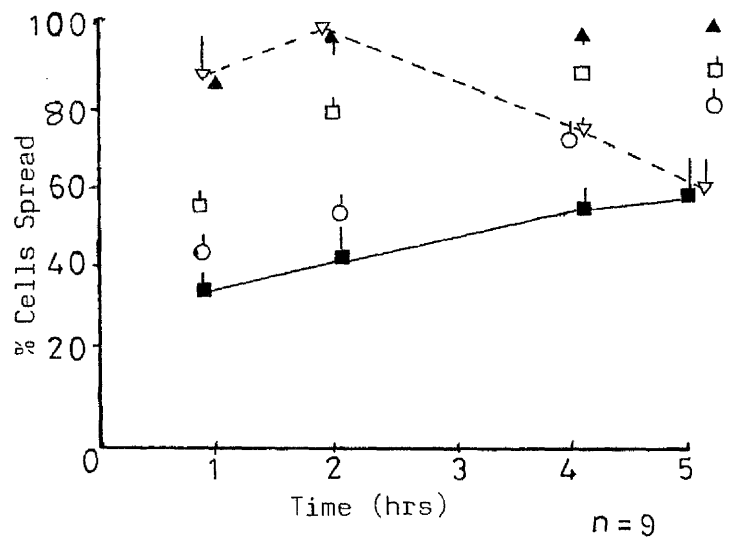

a
b
c
d

FIGURE 20

20a) Effect Of Vanadate On Spreading Of ASV Cells



20b) Reversal Of Vanadate Effects By Washing



Spreading assays were performed as described previously, but ASV cells were used.

20a)

▽ = HS

▲ = 10^{-6}M vanadate

□ = 10^{-5}M vanadate

○ = $5x10^{-5}$M vanadate

■ = 10^{-4}M vanadate

20b)

▽ = HS/10^{-4}M vanadate

▲ = 10^{-6}M vanadate/HS

□ = 10^{-5}M vanadate/HS

○ = $5x10^{-5}$M vanadate/HS

■ = 10^{-4}M vanadate/HS

spreading on any of the cell types investigated. In each case, approximately 95% of cells spread within the first hour of the assay. $1x10^{-5}$M vanadate inhibited spreading in C13 and ASV cells (65% and 40% failed to spread respectively) to a far greater extent than Py cells where approximately 15% of cells remained rounded.

Cells of all types which had been incubated for 2 hours with vanadate and then washed and reincubated in its absence showed a rapid spreading response. 3 hours from removal of vanadate, the proportions of cells spread approached those of controls. This reversal was fastest in the ASV cells (see Figures 18,19 & 20). Washing the cells after 2 hours, and readdition of the same vanadate concentration, caused no appreciable change in the spreading behaviour.

A striking and highly repeatable difference between C13 and the transformed cells was observed when $1x10^{-4}$ M vanadate was added to cells prespread on fibronectin. The addition of vanadate had no effect on the C13 cells. However, addition of $1x10^{-4}$M vanadate to both Py and ASV cells produced an increase in rounded cells. Spread cells rounded up over a period of 3 hours, until the final ratio of spread:rounded cells was approximately the same as that obtained when cells were seeded in the presence of vanadate. Lower vanadate concentrations including one ($1x10^{-5}$M) which did inhibit spreading initially did not appear to cause rounding of spread cells (see Figures 18,19 & 20 and Plates 2,3 and 4).

The short term morphological effects of vanadate appeared to be readily reversible, by washing to remove the ion.

PLATE 4

ASV cells from spreading assay

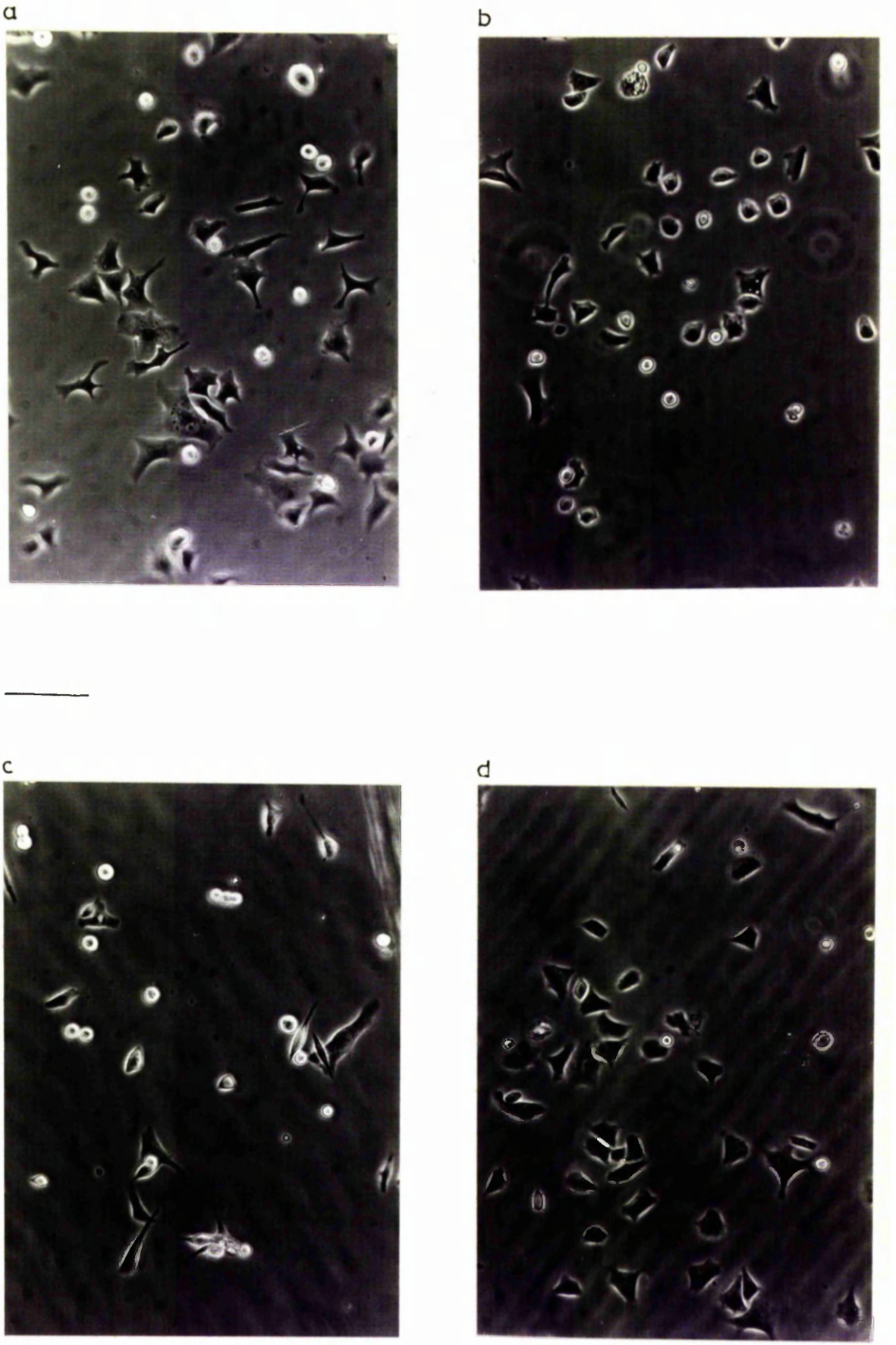
a) Cells spread when incubated in HH.

b) Many cells remained rounded when incubated in the presence of 10^{-4} M vanadate.

c) Many prespread cells began to round up after the addition of 10^{-4} M vanadate.

d) Many rounded cells began to flatten when vanadate was removed by washing.

Bar = 50μm


a
b
c
d

Addition of vanadate to prespread cells caused only the transformed cells to round up within the time course of this assay. It is not clear why the behaviour of C13 cells in these experiments differs from their behaviour in culture since in the presence of serum I have observed C13 cells to round up within 4 hours of addition of $1x10^{-4}$M vanadate. It is unlikely that the presence of serum alone accounts for this difference since in a single experiment the presence of 10% serum did not cause rounding of C13 cells prespread on fibronectin in response to $1x10^{-4}$M vanadate.

Colchicine Experiments

Colchicine ($1x10^{-4}$ M added to coverslips that had not been incubated with vanadate) appeared to slow the spreading of C13 cells, so that after 1 hour about 75% of cells were spread, compared to 95% in controls. These cells had a characteristic 'fried egg' morphology (Plate 5). Within 4 hours, however, values had approached those of controls. With Py cells this effect was more pronounced. After 1 hour about 65% of cells were spread, (83% of cells being spread after 5 hours, this should be contrasted with $1x10^{-4}$M vanadate where 40% of cells remained rounded after 5 hours), (see figures 21a & 21b).

Washing after 2 hours to remove the colchine appeared to make no difference to the proportion of C13 or Py cells spread although the spread cells became more flattened. Addition of colchicine to prespread cells had no effect, over this time course, on C13 cells, but caused a slight rounding up of Py cells, to values similar to those initially obtained when cells

FIGURE 21

Cells were seeded on fibronectin-coated coverslips in the presence or absence of colchicine. After 2 hours the coverslips were washed three times with HH and cells were then re-incbated in the presence or absence of colchicine as indicated.

▽ = HH/ Colchicine

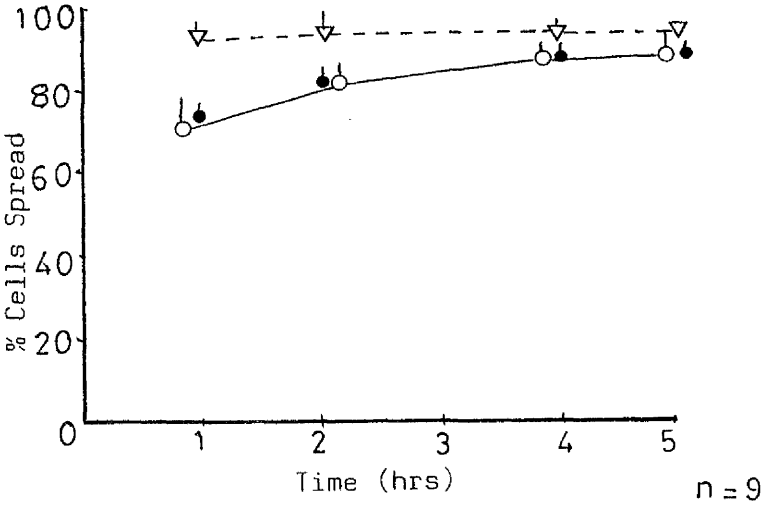
○ = Colchicine/HH

● = Colchicine

Bars represent standard deviation (shown on one side only)

FIGURE 21

21a) Effect Of Colchicine On Spreading Of C13 Cells



21b) Effect Of Colchicine On Spreading Of PY Cells

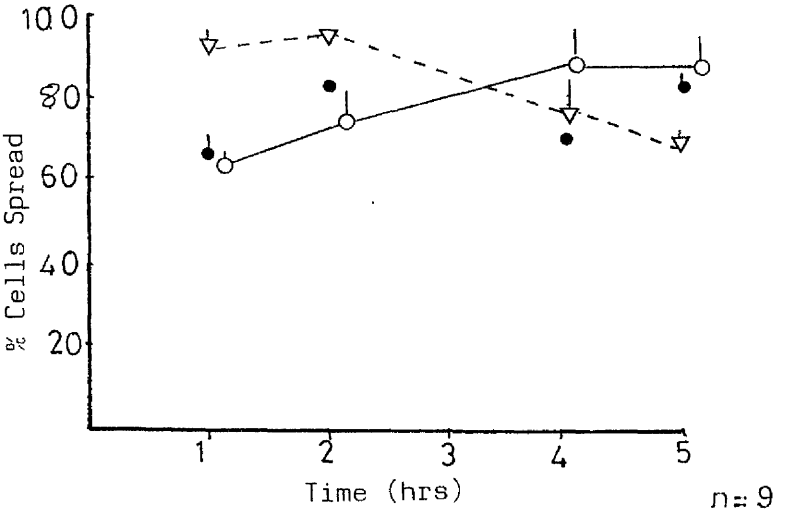


PLATE 5

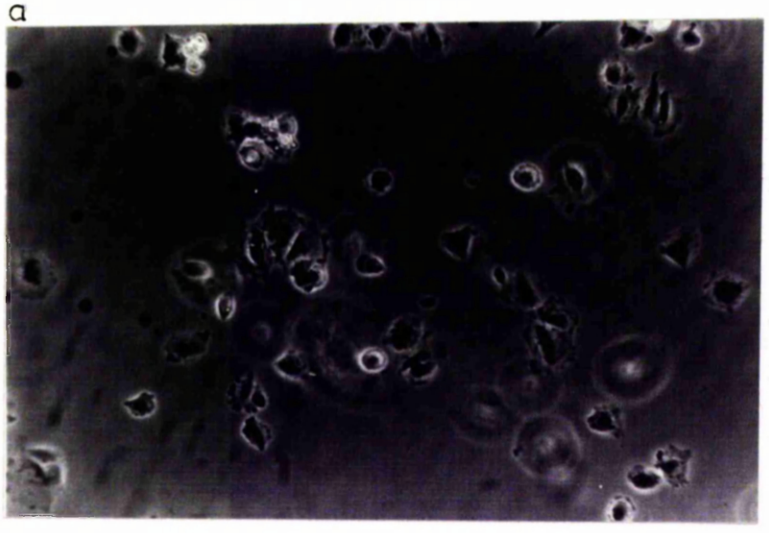
a) C13 cells after 2 hours incubation in HH + 10^{-4}M colchicine.

Note the 'fried egg' morphology.

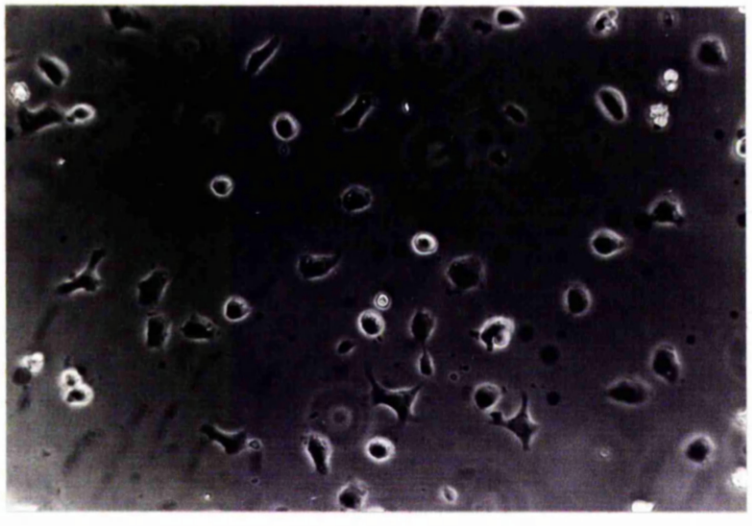
b) PY cells after 2 hours incubation in HH + 10^{-4}M colchicine.

Bar = 50μm

a



b



were seeded in the presence of colchicine.

The morphology of cells spread in the presence of colchicine was quite different to that of the controls. These qualitative changes were not taken into account in the assessment of spreading (Plate 5).

It was surprising that colchicine addition to prespread cells did not alter their morphology. In culture addition of colchicine to semi-confluent monolayers produced the characteristic 'fried egg' shape within 6 hours. The addition of colchicine to prespread cells was investigated for only 3 hours on fibronectin with no serum present. Observation for a longer period may have shown this shape change. Alternatively the lack of response may have been due to the high concentration of FN present on the coverslips in these experiments.

In summary cells seeded onto FN in the presence of vanadate showed an inhibition of spreading of approximately 50%. This inhibition of spreading was still observable after 5 hours incubation in the presence of the inhibitor. Cells seeded onto FN in the presence of colchicine showed an initial inhibition of spreading of approximately 25%. This inhibition of spreading diminished over a period of 4 hours incubation during which time cells eventually spread to levels seen in controls.

Arterenol Experiments

Arterenol or nor-epinephrine is a catechol. These compounds are known to reduce the vanadium(V) species to vanadium (IV), complex it, and thus reverse a variety of effects of vanadate in

FIGURE 22

Cells were seeded on fibronectin-coated coverslips in the presence of vanadate or arterenol as indicated. After 2 hours the coverslips were washed as described previously and cells re-incubated with vanadate or arterenol as indicated.

▽ = HS/Arterenol

○ = Arterenol

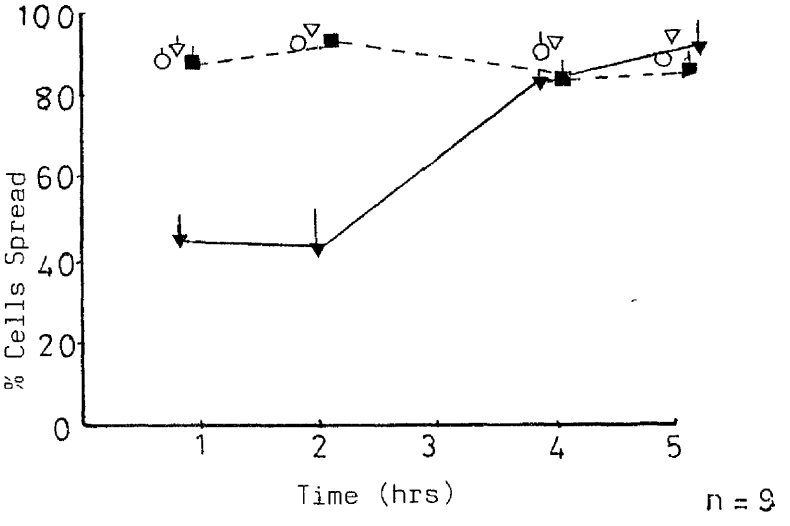
■ = Arterenol/10^{-4} M vanadate

▼ = 10^{-4} M vanadate/Arterenol

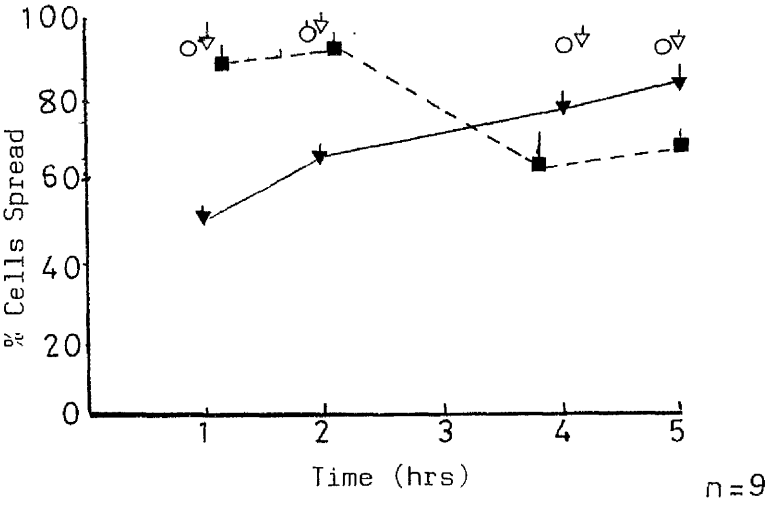
Bars represent standard deviation (shown on one side only)

FIGURE 22

22a) Effect Of Arterenol On Spreading Of C13 Cells



22b) Effect Of Arterenol On Spreading Of PY Cells



vitro (Cantley et al 1978b). Arterenol was used in a series of the spreading/rounding assays in conjunction with $1x10^{-4}$ M vanadate.

In the presence of arterenol, C13 and Py cells assumed a stellate morphology, which was most pronounced with C13 cells. However, this did not effect the proportion of cells spread. Within about 3 hours of addition of arterenol the cells had began to spread out and attain a more normal appearance.

Addition of arterenol to prespread cells had no effect on cell shape. Addition of arterenol to vanadate-treated cells caused a rapid reversion to control values for spread cells. This was apparent within 2 hours, and was most pronounced with C13 cells. Addition of vanadate to cells spread in the presence of arterenol had no effect on the degree of spreading of the C13 cells, but caused a rounding response with Py cells, although it was not as great as previously seen (see Figures 22a & 22b).

Addition of vanadate and arterenol together formed a very dense brown precipitate, outside the cells, through which it was impossible to score the degree of spreading. These experiments indicated that although the effects of vanadate could be reversed simply by washing, the reversion was accelerated by arterenol.

Experiments were then performed to test a number of possible targets for the action of vanadate in rounding cells. These were the Na/K ATPase, adenylate cyclase, the involvement of secretory processes, the effect of cell density, different trypsin treatments and the stage in the cell cycle.

Ouabain Experiments

Vanadate is known to inhibit the Na/K ATPase of intact red blood cells (Cantley et al 1978a). It was decided to see whether inhibition of Na/K ATPase was causing the observed rounding of cells by comparing vanadate with a known inhibitor of this enzyme, the cardiac glycoside ouabain.

Addition of ouabain to cells seeded onto fibronectin caused a small degree of inhibition of spreading (approximately 10%) with both C13 and Py cells. The cells continued to spread in the presence of ouabain, over the time course of the assay, to approach the values obtained for controls. Ouabain did not appear to alter cell morphology. When cells were incubated with ouabain and vanadate together the ouabain did not appear to affect the action of vanadate in any way (Figures 23a & 23b).

Addition of ouabain to prespread cells had no observable effects, and the addition of ouabain to vanadate-treated cells did not enhance, or slow the reversal of inhibition of spreading. Ouabain had no effects comparable with vanadate on either cell type at any of the concentrations tested ($1x10^{-5}$ M - $1x10^{-3}$ M).

Thus it would appear unlikely that the Na/K ATPase was involved in the inhibition of spreading by vanadate.

Another mechanism of action investigated was the possible stimulation by vanadate of adenylate cyclase, which could be expected to raise the cellular cAMP levels. dbcAMP and IBMX a non-hydrolysable analogue of cAMP and a phosphodiesterase inhibitor, respectively, were used to test this possibility.

FIGURE 23

Cells were seeded on fibronectin-coated coverslips in the presence of ouabain and/or vanadate. After 2 hours the coverslips were washed as described previously and cells re-incubated with vanadate or ouabain as indicated

▽ = Ouabain

▲ = Ouabain/10^{-4} M vanadate

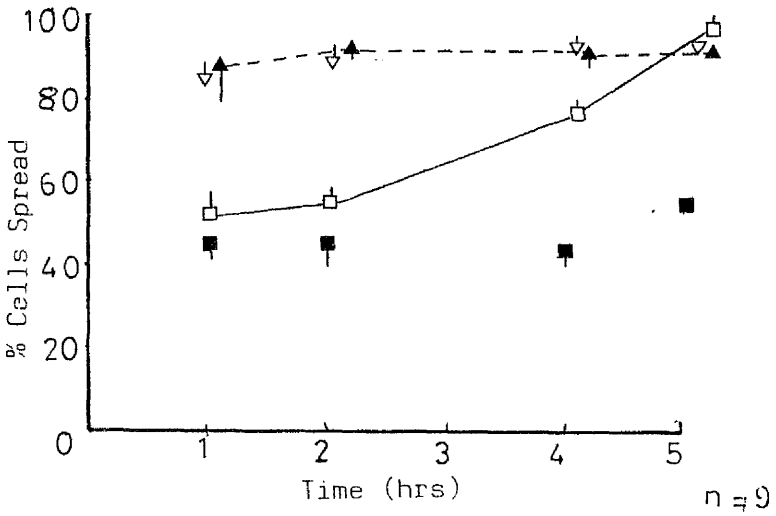
□ = 10^{-4} M vanadate/Ouabain

■ = 10^{-4} M vanadate + Ouabain

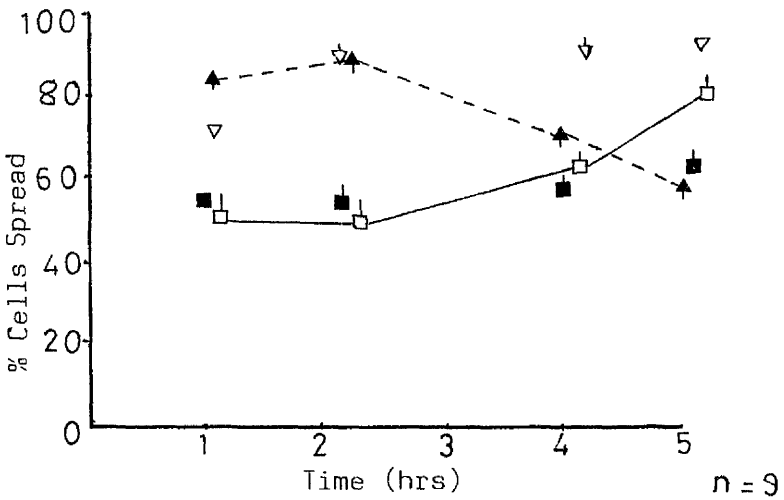
Bars represent standard deviation (shown on one side only)

FIGURE 23

23a) Effect Of Ouabain On Spreading Of C13 Cells



23b) Effect Of Ouabain On Spreading Of PY Cells



dbcAMP and IBMX Experiments

Used in conjuction with $1x10^{-4}$M vanadate and DMSO, a control for the carrier solvent for IBMX, 1µl DMSO was added as described below.

Cells treated with dbcAMP and IBMX, or IBMX alone, assumed a stellate morphology (Plate 6), and the treatment did not alter the proportion of cells spread. After about 2 hours the cells had attained a more normal appearance. DMSO treated cells did not differ from the controls, thus indicating that at the concentration used, DMSO had no effect on the cell morphology.

Addition of dbcAMP and IBMX, IBMX or DMSO to prespread cells caused no change in cell morphology or spreading behaviour. Addition of the above to vanadate-treated cells did not alter the reversal of vanadate inhibition as described earlier. Addition of vanadate to cells spread in the presence of dbcAMP and IBMX, IBMX or DMSO had no effect on C13 cells, but as usual produced rounding of Py cells, in this case approximately 20% of the population (Figures 24a & 24b).

Assuming that the addition of dbcAMP and IBMX did elevate the endogenous levels of cAMP it would appear that the stimulation of adenylate cyclase is not the cause of the inhibition of spreading seen in vanadate- treated cells. The addition of exogenous cAMP has been used to bring about "reverse transformation" in CHO cells (Johnson et al 1971), including an increase in stress fibre orientation and adhesivity to substrate (Hsie and Puck 1971). The radial protrusions responsible for the stellate appearance of the cells in the present work could have been due to an exaggerated expression of stress fibres.

FIGURE 24

Cells were seeded on fibronectin-coated coverslips in the presence of dbCAMP and IBMX or vanadate as indicated. After 2 hours the coverslips were washed as described earlier and cells re-incubated with dbCAMP and IBMX or vanadate.

■ = dbCAMP + IBMX

△ = HS/ dbCAMP + IBMX

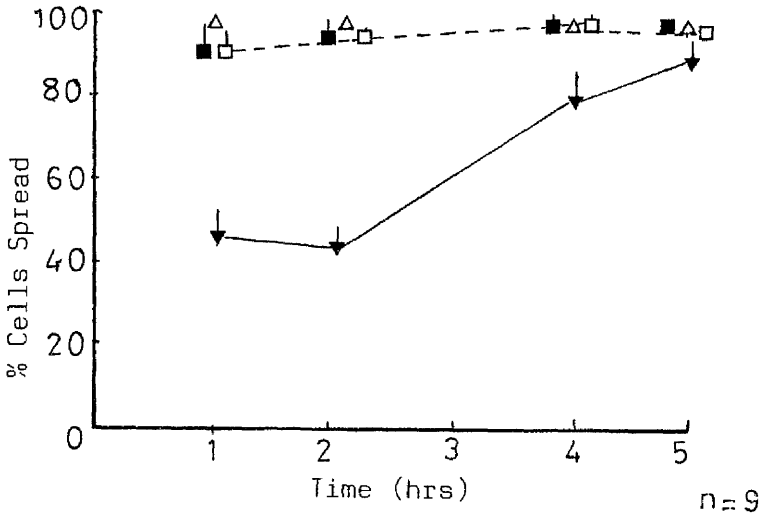
▼ = 10^{-4} M vanadate/ dbCAMP + IBMX

□ = dbCAMP + IBMX/ 10^{-4} M vanadate

Bars represent standard deviation (shown on one side only)

FIGURE 24

24a) Effect Of dbCAMP On Spreading Of C13 Cells



24b) Effect Of dbCAMP On Spreading Of PY Cells

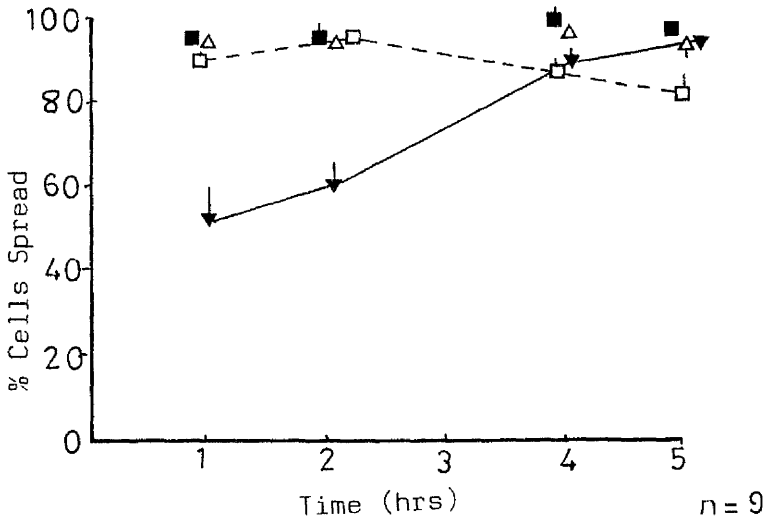


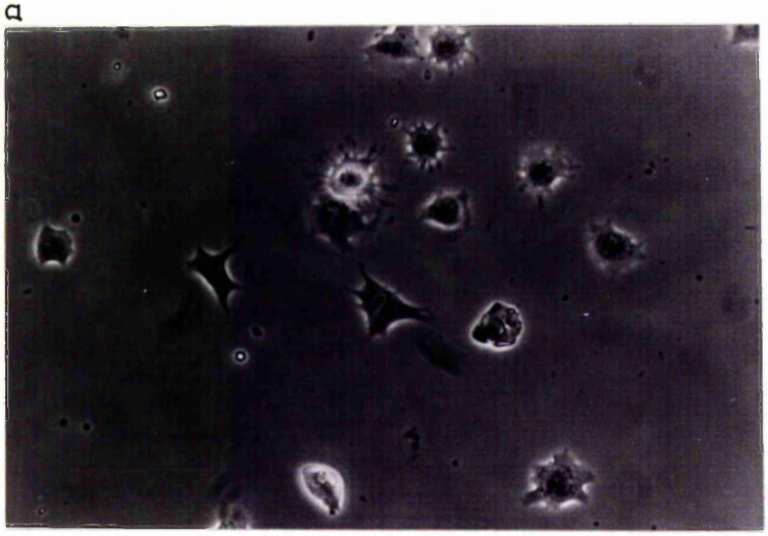
PLATE 6

a) C13 cells after 1 hour in the presence of db CAMP and IBMX.

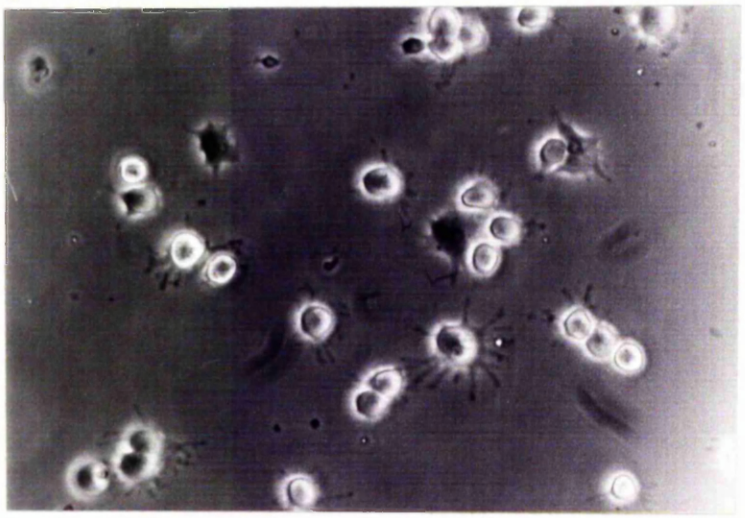
b) PY cells after 1 hour in the presence of db CAMP and IBMX.

Bar = 50μm

a



b



Vanadate Effect On Secretion

A possible target for the effects of vanadate and colchicine on cell aggregation and cell spreading could be the secretion of newly synthesised protein (see discussion).

To monitor protein secretion cells were labelled with ^{35}S Methionine and the rate of release of TCA-precipitable material to the medium was measured in the presence and absence of vanadate and colchicine. Data were adjusted to allow for variation in levels of ^{35}S Methionine in the incubating medium. These results indicate that neither vanadate nor colchicine had any effect on secretion of TCA-precipitable protein in this assay (Figure 25).

These known biochemical effects of vanadate tested did not appear to be instrumental in the observed phenomenon of inhibition of spreading. The next set of experiments was designed to examine parameters that may have been heterogenous in the population, to try to account for the differential effects of vanadate, i.e. why even at $1x10^{-4}$M vanadate some cells were still able to spread.

Effect of Cell Density on Spreading

It was casually observed that if the cell suspension used in the spreading assays contained any cell clumps then the cells in these tended to spread, even in the highest vanadate concentrations, to a greater degree than single cells (see below).

A variety of concentrations of C13 cells, 0.01 million -

FIGURE 25

Cell monolayers were incubated in the presence of HH containing 0.5μCi/ml ^{35}SMethionine for 2 hours, washed and non-radioactive medium (HECT) was added. Vanadate and colchicine were also added as indicated. At 30 minute intervals 100μl samples were taken from the medium and precipitated in 10% TCA. This precipitate was then filtered through 0.22μm Millipore filters in a filter assembly attached to a tap vacuum system.

○ = Control

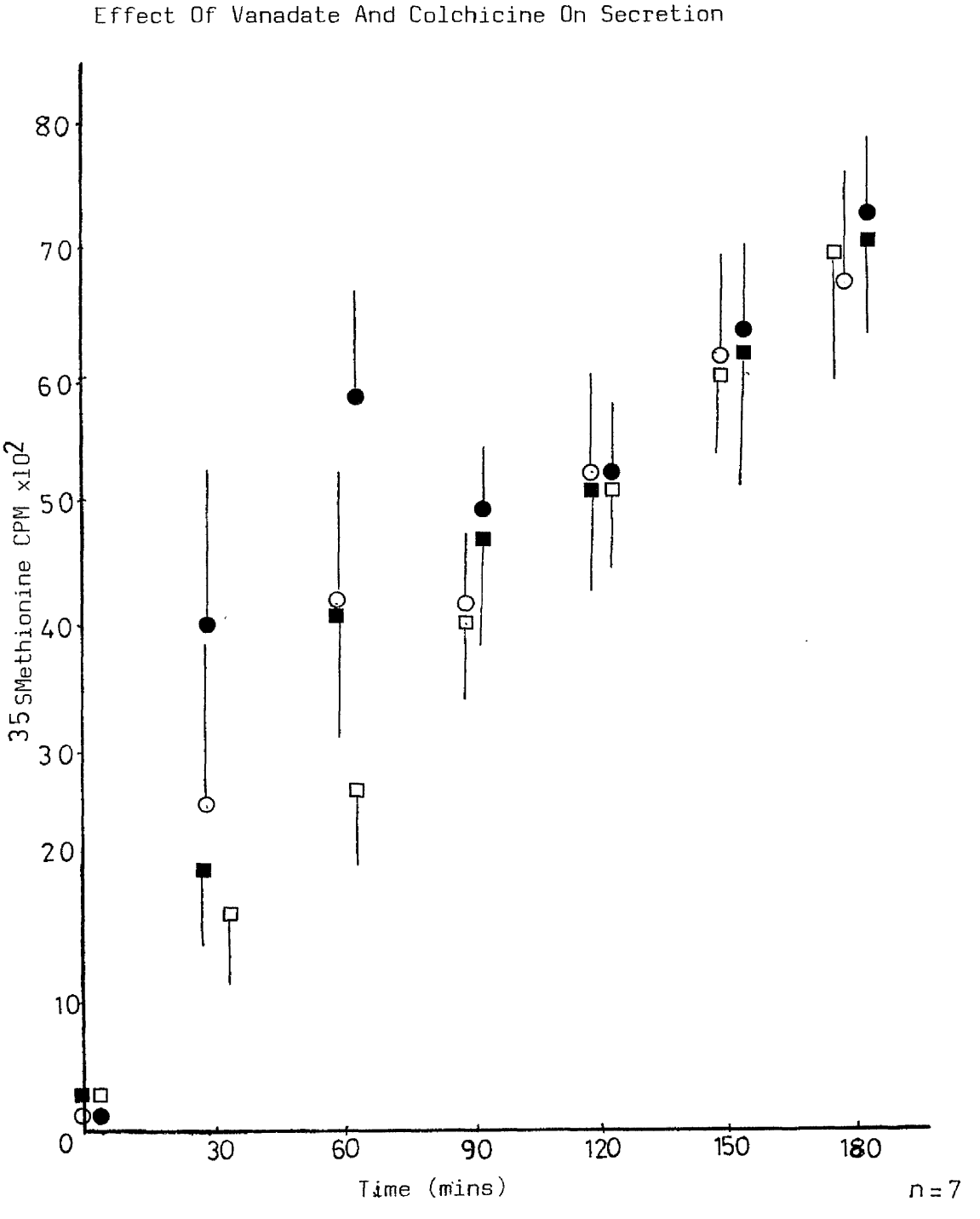
● = 10^{-4}M vanadate

■ = 10^{-5}M vanadate

□ = colchicine

Bars represent standard deviation (shown on one side only)

FIGURE 25


Effect Of Vanadate And Colchicine On Secretion
80
70
60
50
40
30
20
10
0
35SMethionine CPM x10²
30
60
90
120
150
180
Time (mins)
n = 7

0.75 million in HH, were added to FN coated coverslips, in the presence of $1x10^{-4}$M vanadate. After 1 hour the numbers of spread and round cells were counted, in a sweep of fields across the maximum diameter of the coverslip.

From the results shown in Figure 26, it would appear that overall cell density does not affect inhibition of spreading by vanadate, as at all cell densities tested vanadate produced approximately 50% inhibition of spreading. At higher cell densities (1 - 2 million cells per coverslip), it proved impossible to count these as the sheer number of cells precluded a clear view of any individual cell.

Differential Trypsinisation

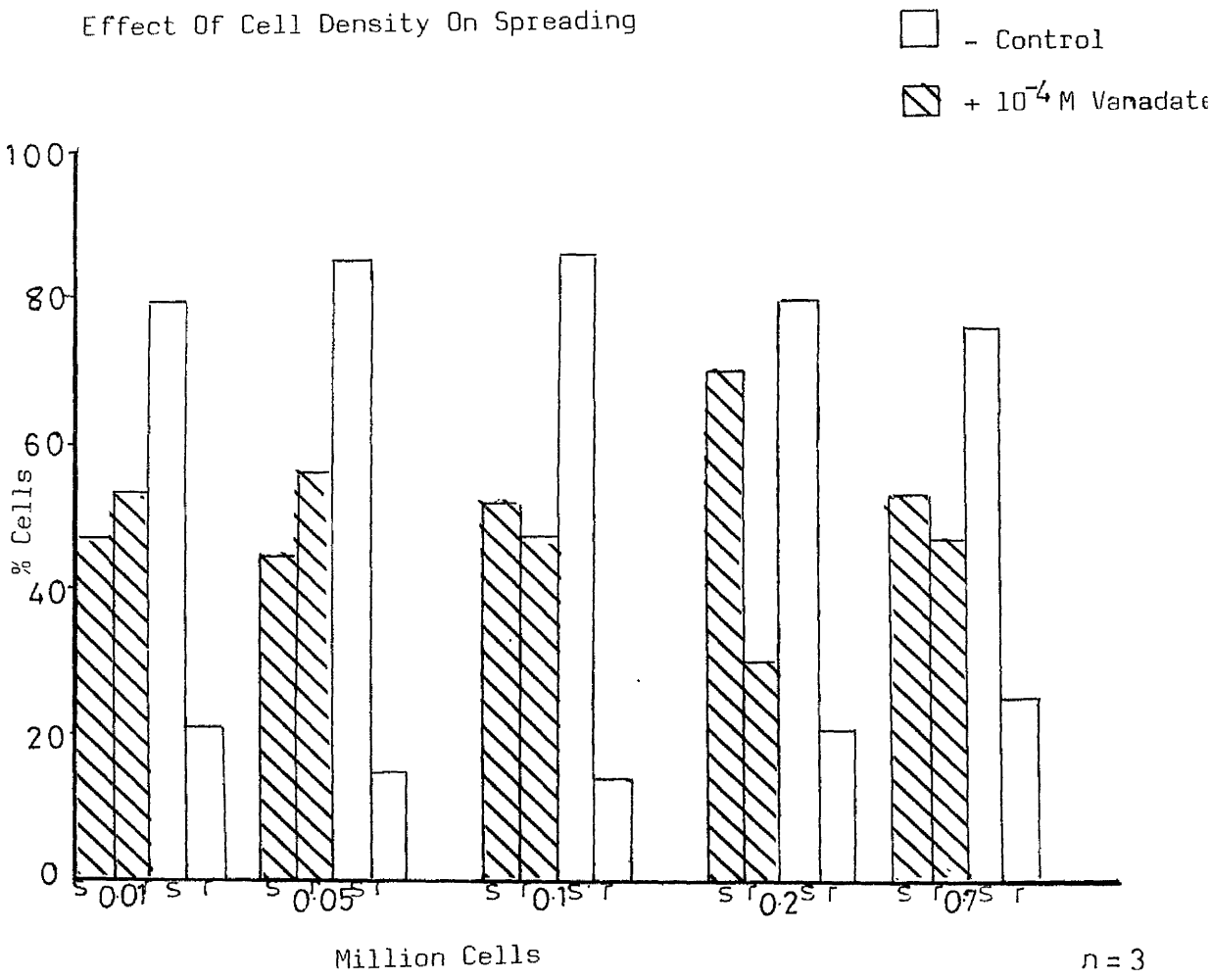
It was possible that the clumps of cells had been protected from mechanical damage in the aspiration of the cell suspensions, or that they had received a lesser trypsin treatment and thus were able to spread more readily than cells depleted of cell surface molecules important in spreading, or that vanadate did not enter to affect cells inside the clumps.

Experiments performed on cells that had been subjected to a range of trypsin treatments indicated that there was no great difference in spreading behaviour of controls, or cells in the presence of $1x10^{-4}$M and $1x10^{-5}$M vanadate, see Table 8.

Effect of Vanadate on Cell Spreading at Different Phases of Cell Cycle

It was thought that cells at different stages of the cell cycle may differ in their sensitivity to rounding by vanadate.

FIGURE 26



Different numbers of cells were seeded on fibronectin-coated coverslips and incubated $\pm 10^{-4}$ M vanadate. After 1 hour the coverslips were scanned along their maximum diameter and the numbers of spread and rounded cells counted. Results expressed as percentage of cells rounded (r) or spread (s).

TABLE 8

Results of spreading assays, in the presence and absence of vanadate, on cells that had had different trypsin treatments.

Trypsin treatment	% Cells Spread (s) And Round (r) Control s	r	$1x10^{-4}$ M [vanadate] s	r	$1x10^{-5}$ M s	r
1.25 mins	92±4	8	67±2	33	72±2	28
2.5 mins	95±1	5	59±7	41	70±4	30
5 mins	95±2	5	54±5	46	66±6	34
20 mins	96±1	4	60±8	40	74±5	26

n = 6

N.B. 5 minutes was the standard trypsinisation for cell harvesting.

^{3}HThymidine pulse/chase experiments were performed to investigate this.

Coverslips were scanned along their maximum diameter and numbers of rounded and spread cells counted. Numbers of radioactive and non-radioactive cells, in each category, were recorded. The percentage of round and spread cells, and the proportion of radioactive cells was calculated.

Cells labelled during the pulse of ^{3}HThymidine will be those that were in S phase of the cycle. This represents about 30% of the total cell population, as is reflected in the results, where for every coverslip counted approximately 25% of the total number of cells were labelled, as shown in Figure 27 and Plate 7.

It was rather surprising that proportions of rounded and spread cells were the same in vanadate-treated coverslips and controls, in view of the inhibition of spreading seen with vanadate (see above). However it was found that TCA treatment caused rounded cells to detach from the coverslips, and that they were subsequently lost. As the same proportions of spread cells were labelled in both controls and vanadate-treated cells, it seems reasonable to assume that had all the rounded cells been retained 25% of them would have been labelled. The data from the spread cells alone indicate that cell cycle differences were not important in vanadate induced rounding.

Immunofluorescence

With C13 cells use of the anti-vimentin antibody showed intermediate filament networks throughout the control cells.

FIGURE 27

Cells were labelled with ^{3}HThymidine (4μCi/ml) for 2 hours, washed and then either used immediately for a spreading assay or incubated with non-radioactive medium for a further 4 hours and then used for a spreading assay.

Cells were then seeded on fibronectin coated coverslips for 1 hour in the presence of various vanadate concentrations as indicated. After 1 hour the cells were processed for autoradiography.

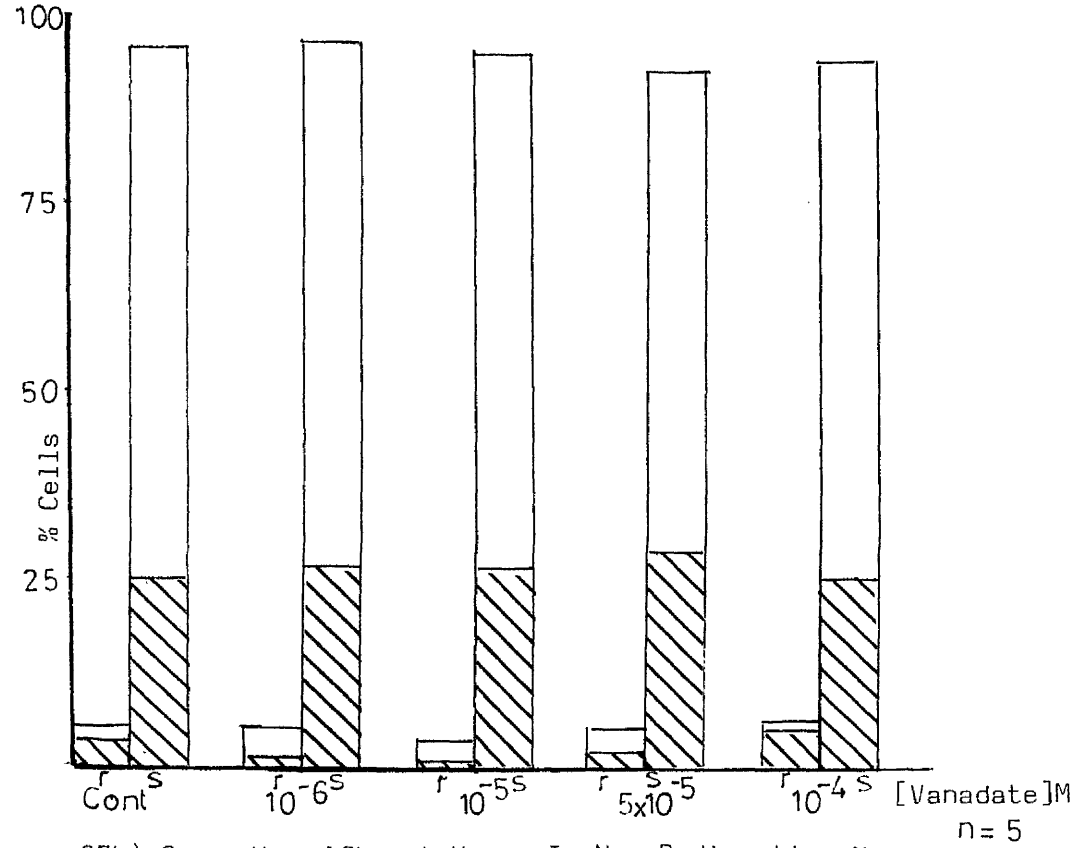
The hatched areas on the graph indicate the proportion of cells with silver grains over their nuclei.

s = Proportion of spread cells

r = Proportion of round cells

FIGURE 27

27a) Spreading Immediately After Labelling



27b) Spreading After 4 Hours In Non-Radioactive Medium

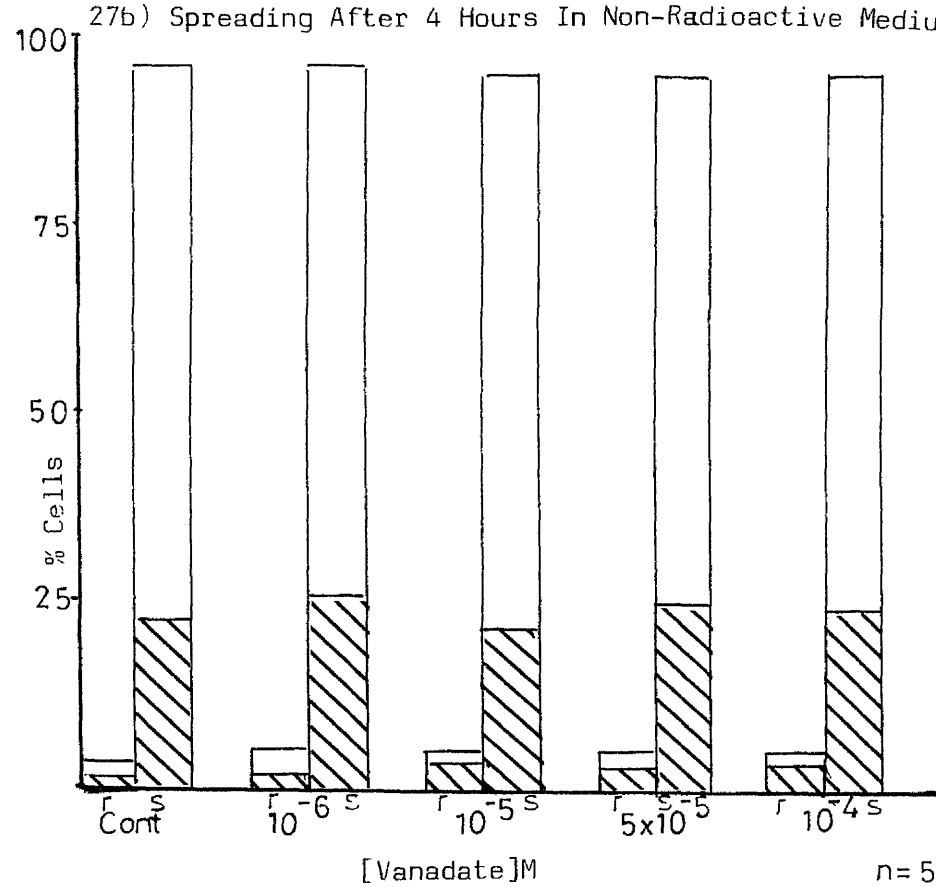


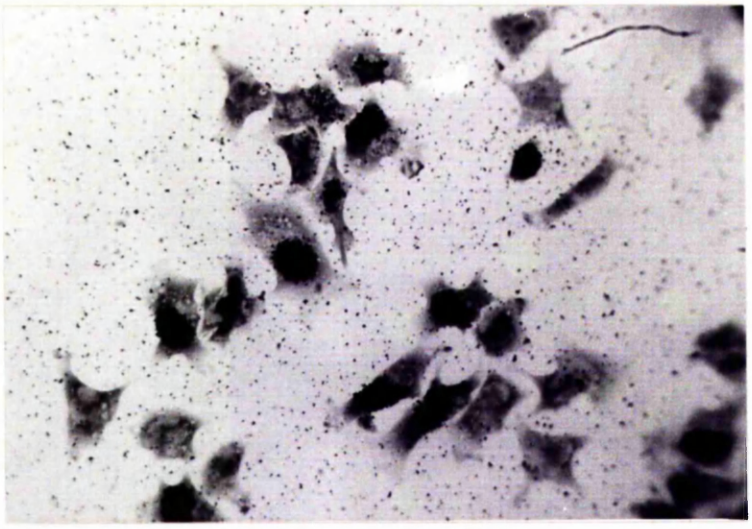
PLATE 7

a) C13 cells processed for autoradiography after spreading on fibronectin-coated coverslips for 1 hour.

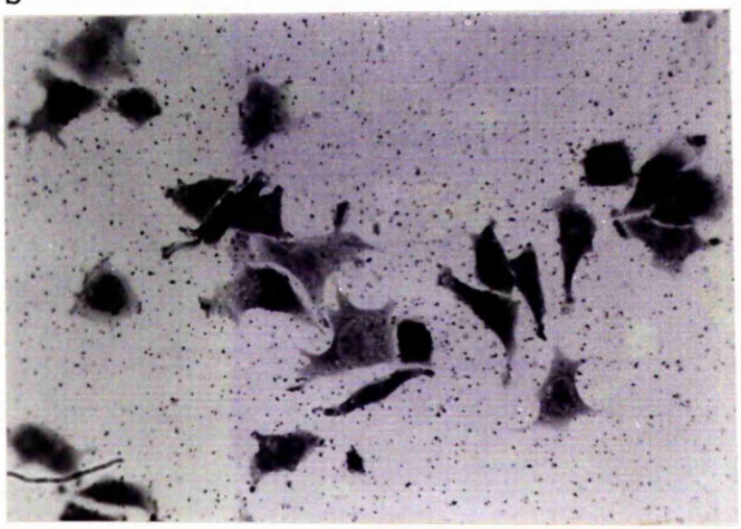
b) C13 cells processed for autoradiography after spreading on fibronectin-coated coverslips for 1 hour in the presence of 10^{-4} M vanadate.

Bar = 20µm

a



b



There was clumping around the nucleus when cells were treated with either colchicine or $1x10^{-4}$M vanadate. This process was observed after 2 hours, was more pronounced after 4 hours and did not appear to be reversible within the time course of the experiment when the inhibitors were removed by washing (see plate 8).

The anti-tubulin antibody was used with C13 cells to visualise the anastomosing networks of microtubules in the control and vanadate treated cells after 2 and 4 hours. In contrast the colchicine treated cells showed bright perinuclear staining after 2 hours. This effect was not reversible after the removal of the colchicine within the time course of the experiment (see Plate 9). This was not surprising in view of the fact that colchicine does not dissociate from microtubules upon washing (Friedkin and Crawford 1983)

The Rhodamine-Phalloidin conjugate was used to show the distribution of actin-containing stress fibres in the C13 cells. The stress fibres formed an ordered array within the control cells. After 2 and 4 hour incubations in colchicine or vanadate the stress fibres appeared to have become laterally aggregated to a small degree. This lateral aggregation could have been due to the shape changes of the cells during these treatments, i.e. cells incubated in vanadate became needle-shaped and those incubated in colchicine became more stellate (see plate 10). The stress fibres in the vanadate treated cells were no longer straight, almost as if an anchorage at each end had been released (see Plate 10b). This is very interesting in view of the involvement of vinculin in actin anchorage and the possible phosphorylation of vinculin upon transformation and by vanadate

PLATE 8

Indirect Immunofluorescence of IF Displays in C13 Cells

a) Indirect immunofluorescence to visualise the IF display in C

Bar = 20μm

b) Effect of vanadate on IF in C13 cells spread on FN.

Bar = 15μm

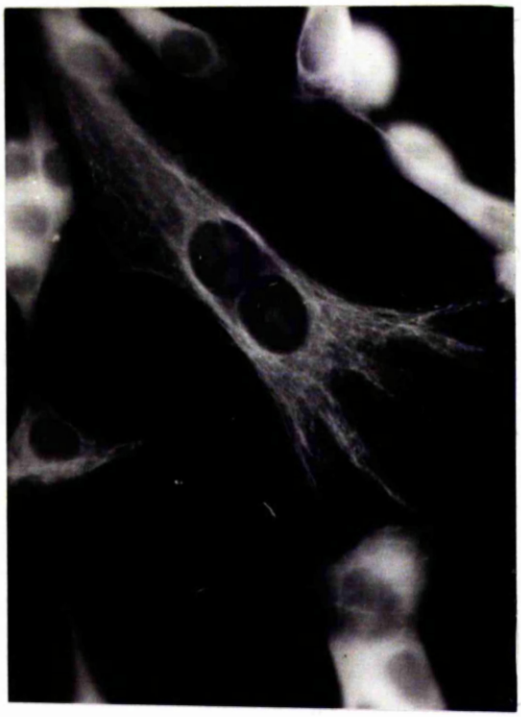
c) Effect of colchicine on IF in C13 cells spread on FN.

Bar = 20μm

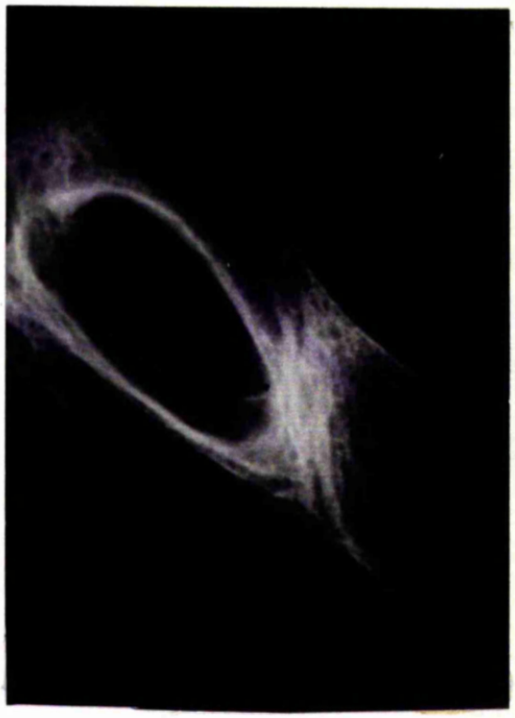
d) Negative controls, no anti-vimentin antibody added.

Bar = 20μm

a



b



c

d

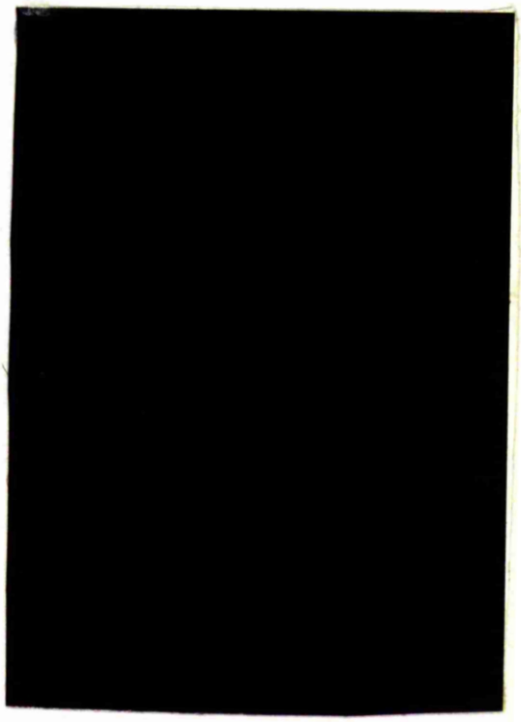


PLATE 9

Indirect Immunofluorescence of Microtubule Displays in C13 Cells

a) Indirect immunofluorescence to visualise the microtubule displays in C13 cells spread on FN.

b) Effect of vanadate on mt displays in C13 cells spread on FN.

c) Effect of colchicine on mt displays in C13 cells spread on FN.

d) Negative controls, no anti-microtubule antibody added.

Bar = 15μm

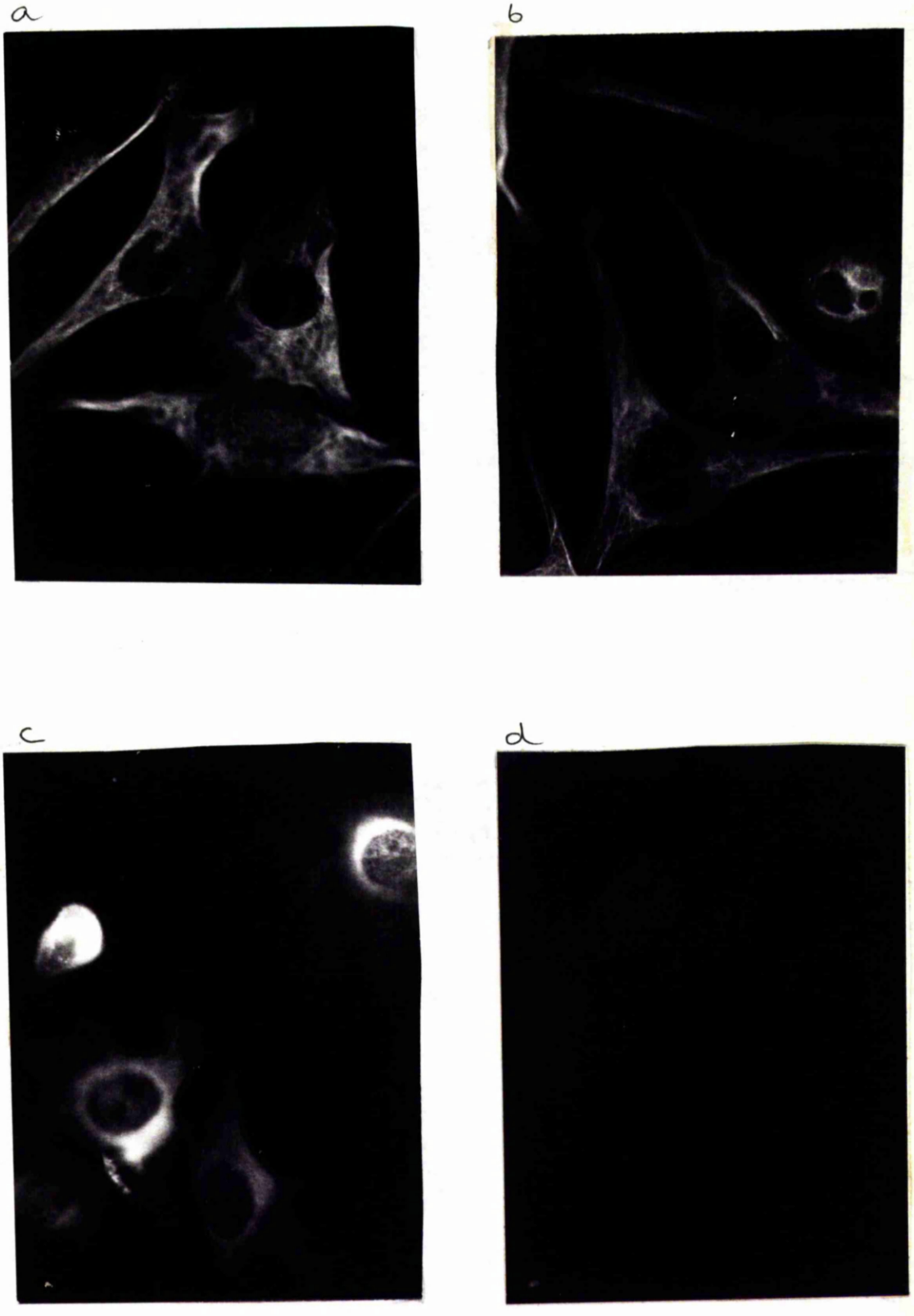

a
b
c
d

PLATE 10

Actin Distribution in C13 cells

a) Stress fibre display in C13 cells spread on FN.

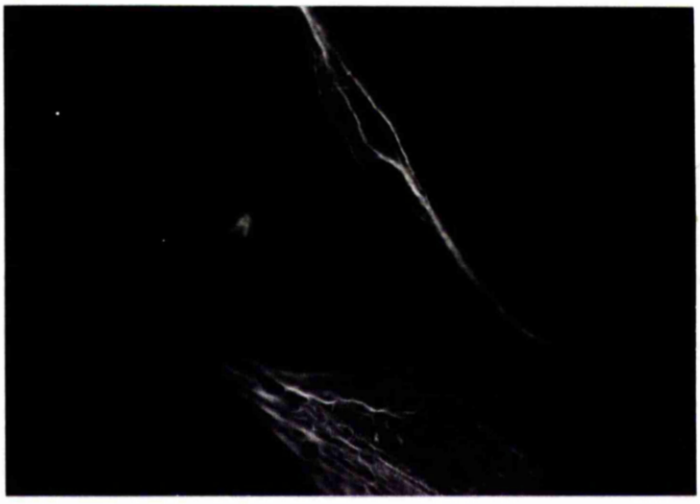
b) Effect of vanadate on the actin distribution of C13 cells spread on FN.

c) Effect of colchicine on the actin distribution of C13 cells spread on FN.

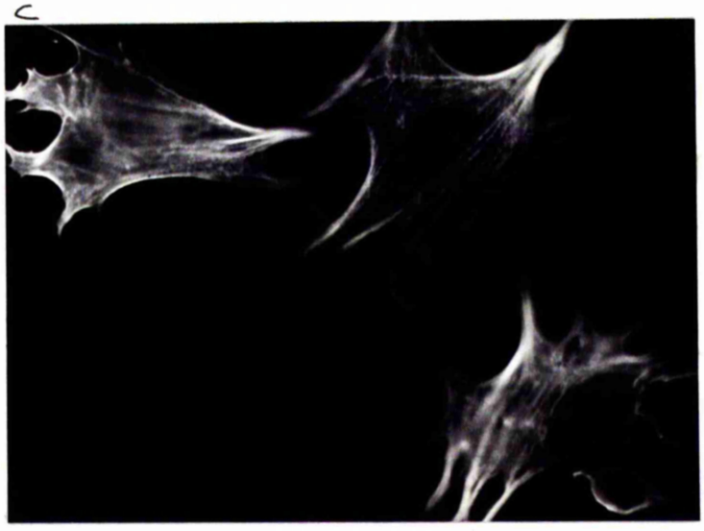
Bar = 20μm

a

b



c



treatment (see discussion).

Py cells were used for immunofluorescence using the Rhodamine-Phalloidin conjugate only. This was due to difficulties because of the rounded nature of these cells, even after spreading on FN. Controls were flat enough to observe intermediate filament and microtubule networks throughout the cells. However colchicine and vanadate treated cells were too rounded to obtain a reliable idea or picture of the distribution of fluorescence. The processing, e.g. acetone at -20°C, caused the majority of cells to be lost from the coverslips, the transformed cells having less tenacity of attachment to the coverslips than their non-transformed counterparts.

The Rhodamine-Phalloidin conjugate showed that the stress fibres were not ordered in the control Py cells compared to the control C13 cells. There was a diffuse filamentous staining in the Py cells. The vanadate treatment disrupted the actin arrays; fluorescence was observed around the cell nucleus. The onset of this was fairly rapid (within 2 hours). The colchicine also disrupted the stress fibres. As with the vanadate treatment fluorescence was observed at the cell nucleus and in addition there was a dot-like distribution of the fluorescence in the cell (see plate 11). This was observable within 1 hour. Neither the vanadate nor the colchicine effects on the actin distribution were reversible within the time course of the experiment. The results were hard to interpret due to the rounding and loss of the majority of the cells during processing. Scattered all over these coverslips small "footprint" areas were observed. These were small areas where stress fibres appeared to have been left attached to the coverslip, the remainder of the cell having been

PLATE 11

Actin Distribution in Py cells

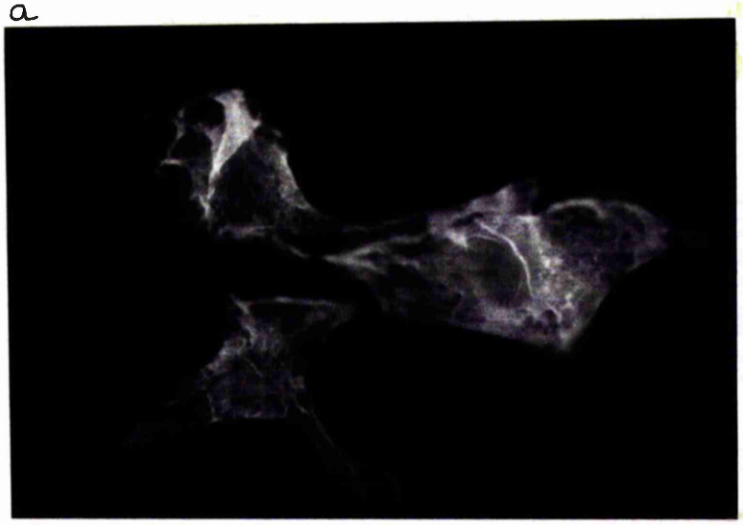
a) Actin distribution in Py cells spread on FN.

b) Effect of vanadate on actin distribution in Py cells spread on FN.

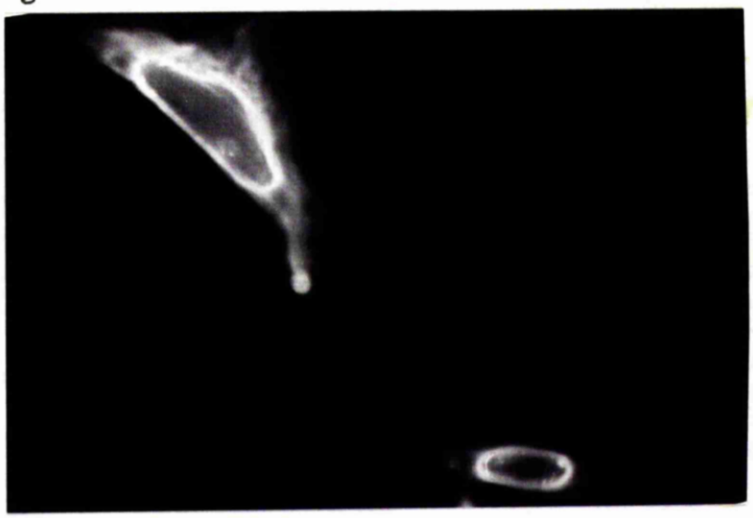
c) Effect of colchicine on actin distribution in Py cells spread on FN.

Bar = 20μm

a



b



c

lost.

The loss of organisation of stress fibres in these vanadate-treated transformed cells was not as marked as some instances reported (e.g. Hanafusa 1977; Shriver and Rohrschneider 1981) but was nevertheless quite obvious (see plate 11). Erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA), an inhibitor of dynein ATPase, thought also to have actions similar to cytochalasin has also been shown to induce dramatic changes in the actin organisation of cultured cells (Schliwa et al 1984). EHNA treated cells displayed dot-like structures in the cytoplasm when stained with Rhodamine-Phalloidin. In this case the cells remained flattened and the dot-like structures reassembled into stress fibres once the EHNA was removed (Schliwa et al 1984), unlike the cells in this study, where the colchicine effect was not reversible. The dot-like structures seen in this work were fewer and larger than those observed in EHNA treated cells (See Figure 11c and Schliwa et al 1984).

The results obtained by immunofluorescence indicate that the intermediate filament display in C13 cells was altered by both vanadate and colchine, whereas the microtubule display was disrupted by colchicine and remained intact in the presence of vanadate. The transformed Py cells have an altered actin distribution when compared to the non-transformed C13 cells (see plates 10 & 11). Vanadate and colchicine, which cause the transformed cells to round up have been shown to alter the actin distribution within these cells far more dramatically than in the C13 cells (plate 10). However C13 cells plated in the presence of vanadate or colchicine, that remained rounded, may have been lost during the processing and thus their stress fibre

PLATE 12

Hoechst 33258, DNA stain

a) Nuclei of C13 cells in culture. Interphase nuclei clearly seen. Bar = 20µm

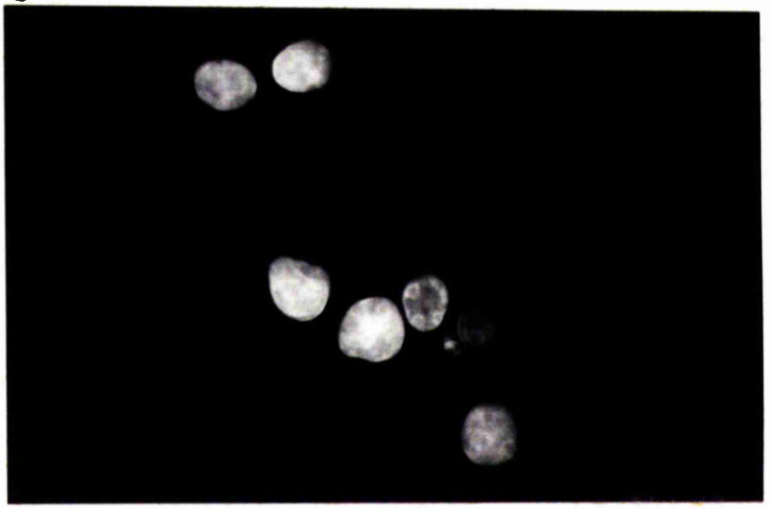
b) Effect of vanadate on C13 in culture. Interphase nuclei clearly seen. Bar = 20µm

c) Effect of colchicine on C13 in culture. Chromosomes clearly seen. Bar = 10µm

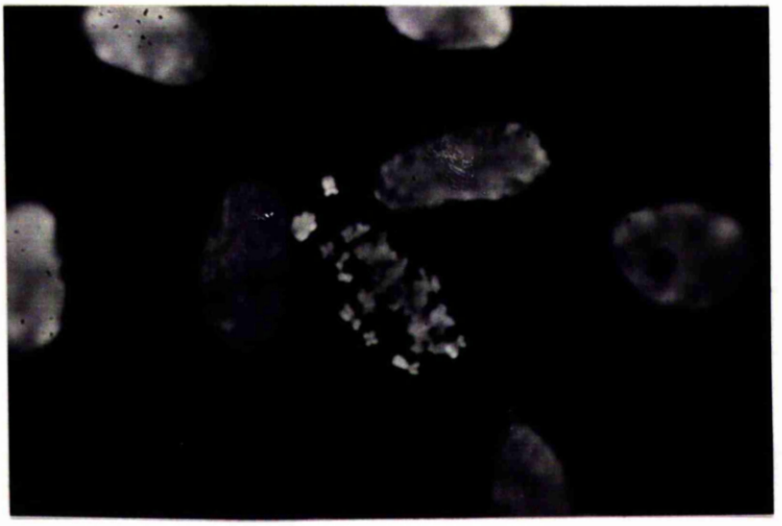
Hoechst 33258 is also used to routinely screen cultures for mycoplasma contamination. These micrographs indicate that the cultures used during this work were free from mycoplasma infection.

a

b



c



distribution may have been altered and escaped detection. The effects of vanadate and colchicine, on the distribution of stress fibres, intermediate filaments and microtubules, were not reversible within the time course of the experiment once the inhibitors were removed. However in the spreading assays performed the rounding effects of these inhibitors were reversible under identical conditions.

Does Vanadate Cause Metaphase Block?

The Hoechst 33258 fluorochrome stained interphase nuclei in control and vanadate treated cells (Plate 12a and 12b). In colchicine treated cells, where the microtubule poison blocks cells in metaphase of mitosis, chromosomes could be made out distinctly in 20% of cells (Plate 12c). Since vanadate did not cause accumulation of cells in mitosis this would indicate that although colchicine and vanadate produce accumulations of rounded cells they do so by different mechanisms.

Effects Of Vanadate On Cell Morphology:Electron Microscopy

SEM

Micrographs obtained by SEM illustrate changes in cell surface morphology after various vanadate treatments. Control cells appeared flattened and relatively smooth (Plate 13a). Rounded cells, presumably those in division, had blebbed surfaces. After 2 hours incubation in the presence of $1x10^{-4}$ M vanadate (Plate 13b) the cells had a similar surface morphology to those of the controls. Some of the cells appeared to be less well flattened, as if rounding up. After 6 hours

PLATE 13

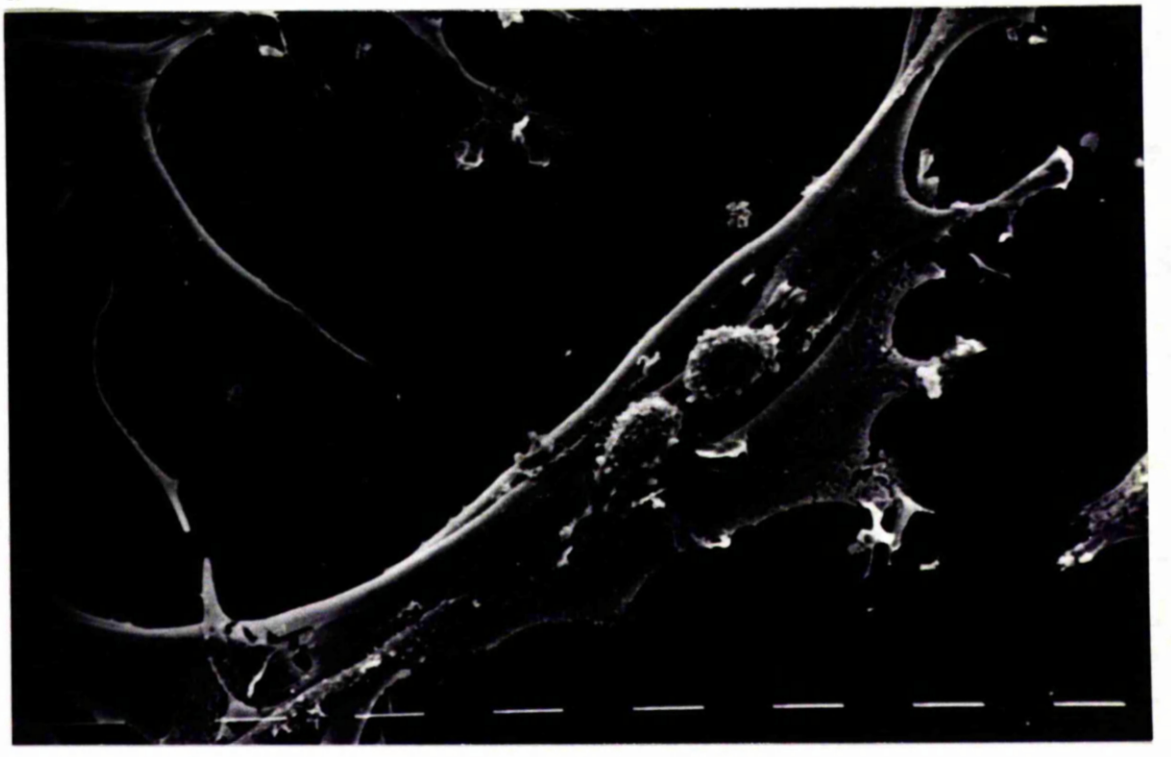
SEM micrographs of C13 cells incubated in 10^{-4} M vanadate for different times.

a) Controls, no vanadate added. Note the flattened appearance of the spread cells and the blebbed, rounded mitotic cells. Surface cracking due to damage from critical point drying.

b) 2 hours vanadate. Note the flattened appearance of the spread cells and the blebbed and microvillous rounded cells. The rounded cell in the foreground was probably damaged during processing.

Bars on micrographs = 10μm

a



b

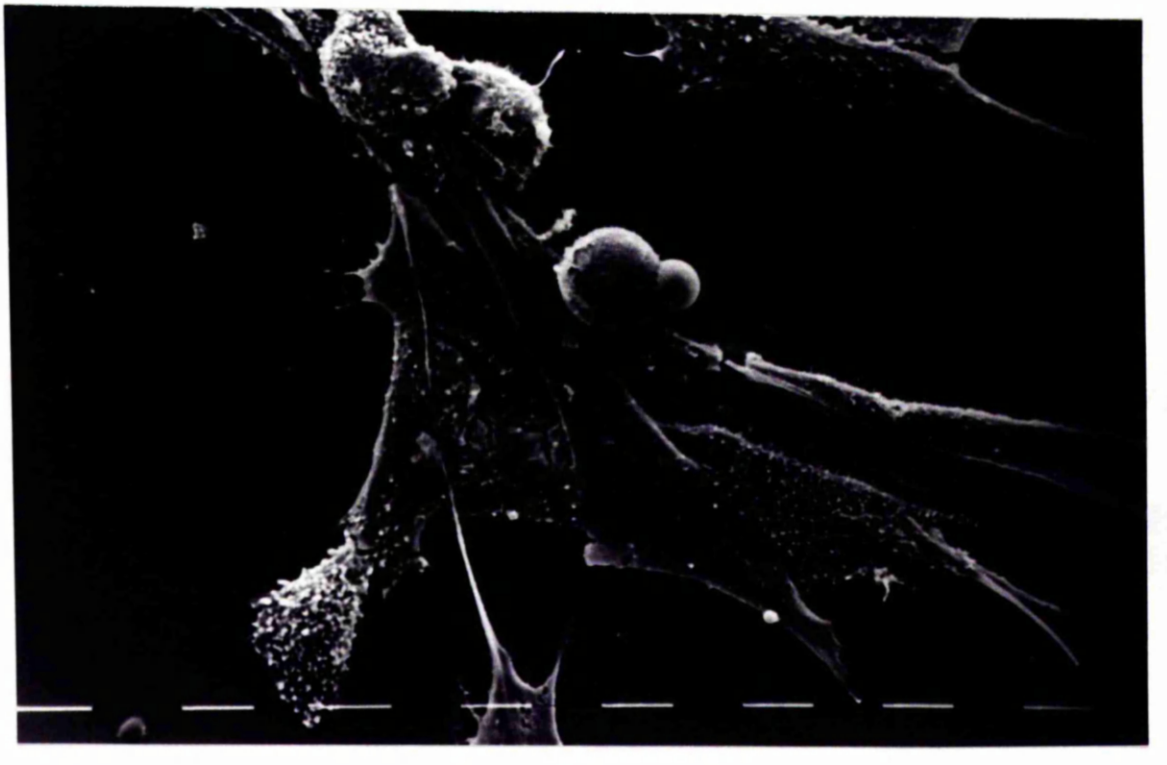


PLATE 14

SEM micrographs of C13 cells incubated in the presence of 10^{-4} M vanadate for different times.

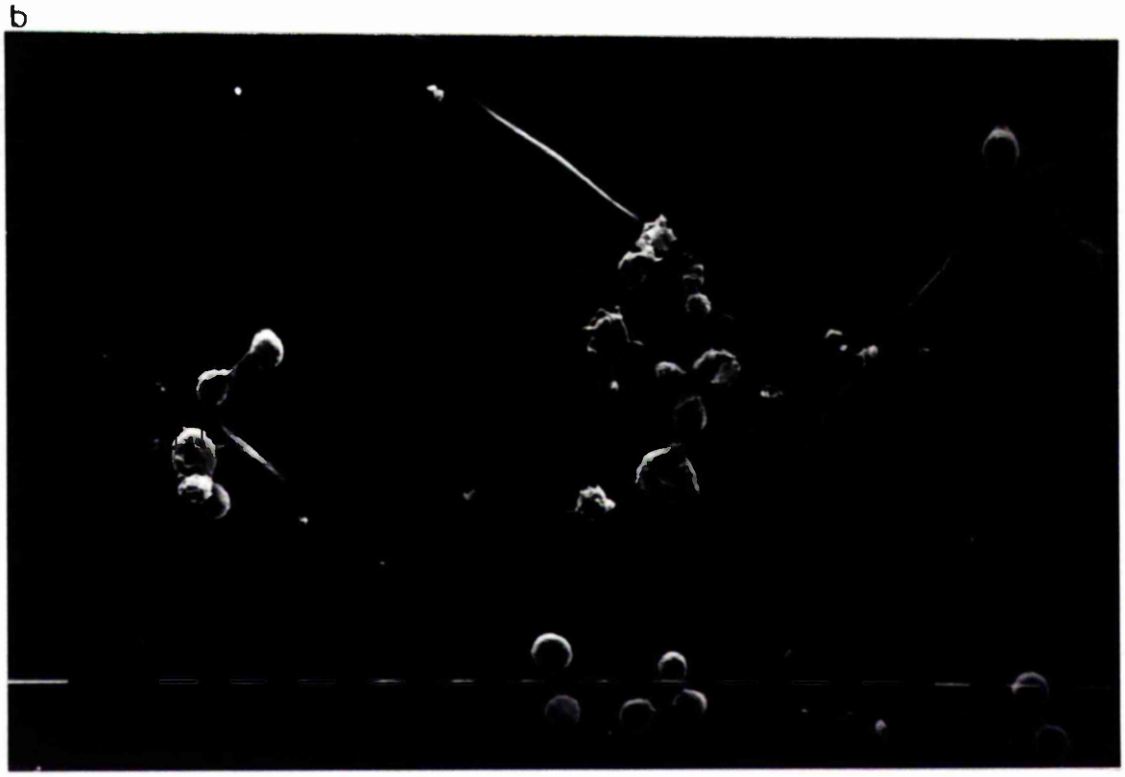
a) 6 hours vanadate. There were fewer spread cells and an increase in the number of blebbed, rounded cells.

b) 18 hours vanadate. The remaining spread cells were needle-shaped and very poorly flattened. The large numbers of vanadate-induced rounded cells were smooth, indicative of dead or dying cells.

Bars on micrographs = 10μm

a

b



incubation with $1x10^{-4}$M vanadate there were more rounded cells present (Plate 14a). After 18 hours incubation in the presence of $1x10^{-4}$M vanadate there were very few flattened cells present. Those that remained were needle-shaped and smooth (Plate 14b). There were many rounded cells that appeared to be smooth, with very little surface topography. This feature is often indicative of dead or dying cells (T.D. Allen personal communication).

TEM

In ultrathin sections observed by TEM, control cells (Plate 15) had regular shaped nuclei and many of the characteristic organelles present. After 6 hour incubation in $1x10^{-4}$M vanadate the nuclei of some of the cells appeared to be highly indented. This was more accentuated after 18 hour incubations in the $1x10^{-4}$M vanadate (plate 16). Approximately 35% of cells displayed these indented nuclei on any one grid observed.

The TEM evidence suggests that there are some gross morphological changes of the nuclei in vanadate-treated cells. Many other organelles appear to be morphologically unchanged.

Does Vanadate Effect Cytoskeletal Protein Insolubility?

Cytoskeletal residues from cells incubated in the presence of $1x10^{-4}$ M vanadate were run on an SDS polyacrylamide gel to determine whether there were any changes in the pattern of Triton-insoluble proteins. The insoluble residues were run in one dimension only and showed no differences between treated and control cells (Plate 17). N.B. Charge changes in the proteins could not be detected by this method. Thus alterations in the state of phosphorylation of these proteins were not detected.

PLATE 15

TEM of trypsin dissociated, pelleted C13 cells. Note the regular shaped nucleus.

Bar = 3μm

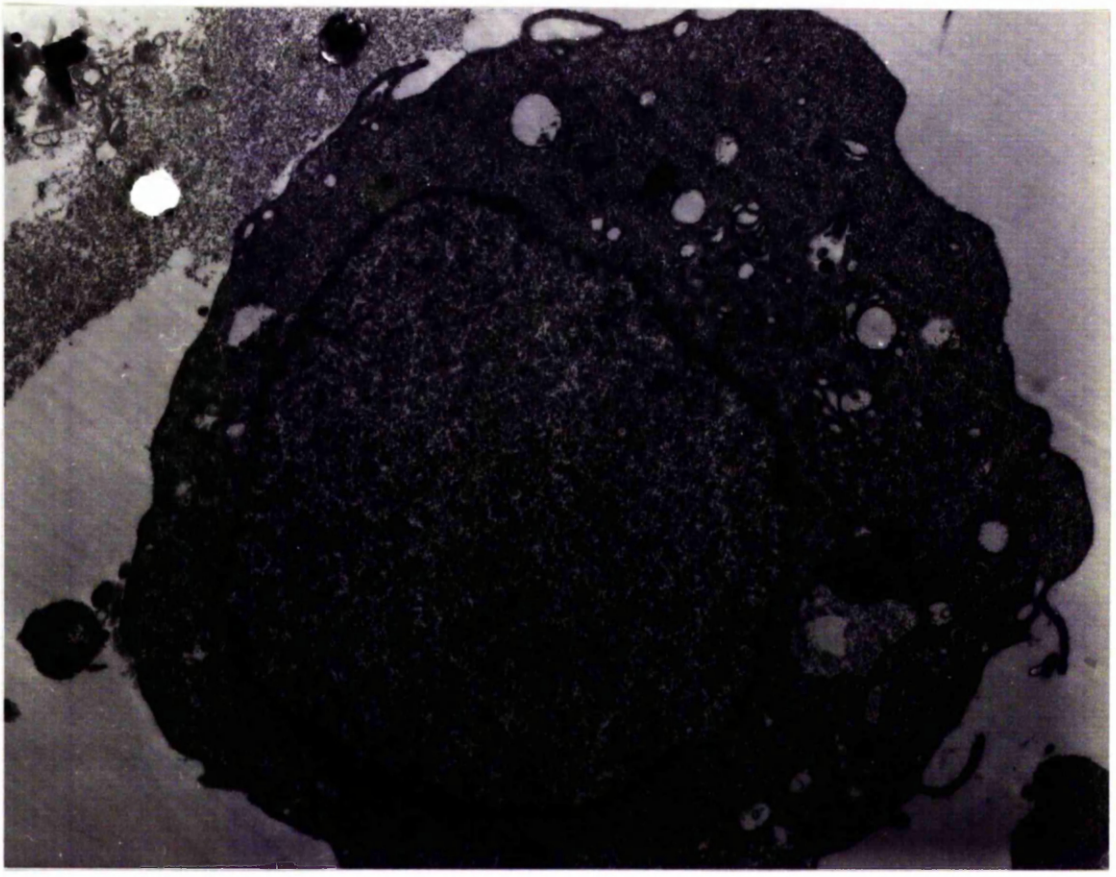


PLATE 16

TEM of trypsin dissociated pelletted C13 cell after an 18 hour pretreatment with $1x10^{-4}$ M vanadate. Note the highly irregular and indented nucleus.

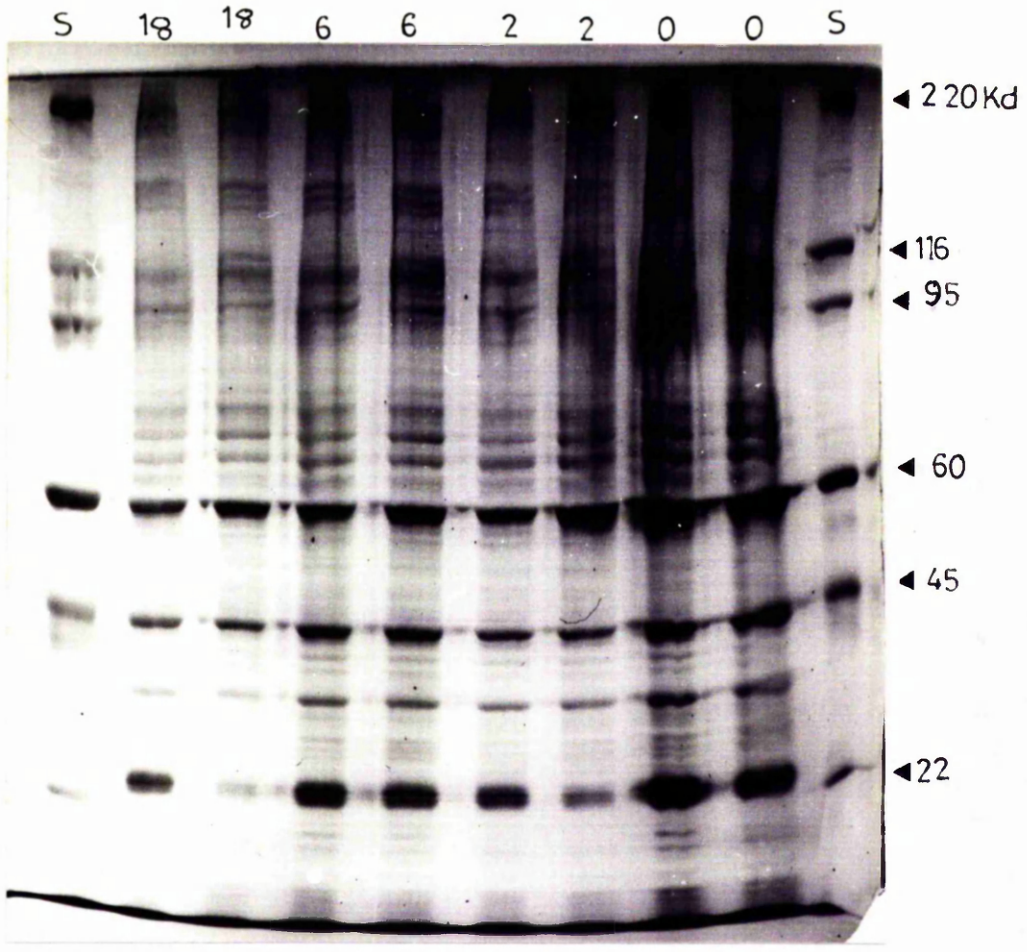
Bar = 3μm

PLATE 17

Analysis of detergent insoluble residues by SDS-PAGE.

Detergent insoluble residues of C13 cells that had been incubated for 0,2,6 or 18 hours in the presence of 10^{-4} M vanadate, as indicated above each track. 30µl of each sample was loaded onto a 6%-10% gradient gel. The gel was stained with Kenacid blue.

Molecular weight markers used (tracks marked S) are described in materials section.


S
18
18
6
6
2
2
0
0
S
220 Kd
116
95
60
45
22

DISCUSSION

In this laboratory two reversible effects of vanadate on live intact cells have been observed: 1. Inhibition of aggregation (Edwards unpublished results), 2. Inhibition of spreading (this Thesis).

Inhibition of cell aggregation by vanadate was previously shown to be reversible (by Edwards unpublished results). My work has demonstrated the reversible inhibition of cell spreading by vanadate. The dose response of the two systems are similar. $1x10^{-5}$ M vanadate produced half-maximal inhibition of aggregation (Edwards 1981) and half-maximal inhibition of spreading of C13 cells (see Figure 19). This similarity may simply reflect the concentration-dependence for the uptake of the ion. Alternatively it may indicate that there is a common mechanism for both reversible effects. For comparison, redistribution of IF has been observed at concentrations between $1x10^{-7}$ M - $1x10^{-5}$ M vanadate (Wang and Choppin 1981). However in my work redistribution was observed at vanadate concentrations of $5x10^{-5}$ M and above. If these effects are mediated internally the vanadate must enter the cell.

I propose to discuss the following:

1. Whether vanadate enters cells and the resultant internal vanadate concentration.

2. Possible mechanisms by which vanadate inhibits the spreading and aggregation of intact cells.

1.Vanadate Uptake

I shall argue in this section that there is evidence, from my work and that of others, that vanadate does enter the cell, but that it is present in lower concentrations than outside (possibly reduced to the vanadium 4+ vanadyl form).

a). Mechanism of uptake

My results have shown that there appears to be some specific uptake of ^{48}V-Vanadate by live cells (See Fig 11b) to values about ten times that expected if equilibrium were to be reached across the cell membrane. The experiments using ^{32}P-Phosphate indicated that the vanadate did not compete for phosphate entry sites (see Figs 9, 10). This latter observation is in accordance with the results obtained by Werdan et al (1980) using rat heart cells. However in intact erythrocytes Cantley et al (1978a) found that vanadate did compete for phosphate entry sites. It has been proposed that due to the differences in the stability of the inner sphere of atoms bound directly to the metal, vanadates and phosphates are unlikely to have similar mechanisms of uptake other than the simplest anion transport system (Rubinson 1981).

In erythrocytes, where pelleted intact cells suspended in medium containing ^{48}V-vanadium were used, there appeared to be an initial fast rate of entry of vanadate that was energy independent (Cantley et al 1978a). Similarly in rat heart fibroblasts and rat heart muscle cells , where cell monolayers were washed and dissolved with NaOH after incubation in medium containing ^{48}V-vanadium, initial vanadate uptake was shown to be energy independent (Werdan et al 1980). It was argued that

because the affinity of vanadate for the intact erythrocytes was 3 orders of magnitude greater than for the isolated Na/K ATPase, the vanadate had to cross the membrane (Cantley et al 1978a).

Further evidence has been obtained that has been interpreted to mean that the vanadate is taken into the cell and is not bound to the external surface of the membrane. The initial phase of vanadate uptake into erythrocytes is rapid (t 1/2 = 4 minutes). After this initial rapid uptake the internal concentration of vanadate equals that of the external environment (Cantley et al 1978a). During the subsequent slower phase of uptake (t1/2 = 90 minutes) the internal ^{48}V-Vanadium is concentrated to levels over 10x that found externally. Addition of arterenol during this second phase did not cause a reduction in cell-associated vanadium or reverse the inhibition of the Na/K ATPase in intact erythrocytes. The arterenol did reverse the inhibition, by vanadate, of the isolated enzyme (Cantley et al 1978a).

Experiments have shown that vanadate ($4X10^{-5}M$) binding to haemoglobin was quite slow t 1/2 = 90 minutes in intact erythrocytes. The ^{48}V-vanadium was then associated with the blood pigment and only a small percentage of it passed through a membrane filter (Cantley et al 1978a). The addition of the anion exchange inhibitor SITS (see below) has been shown to slow the onset of inhibition of the Na/K ATPase suggesting that the uptake of vanadate was essential for its inhibition (Cantley et al 1978a).

Rossi et al (1981) used erythrocyte ghosts resealed in vanadate containing medium which were then incubated in vanadate-free medium. These ghosts showed an inhibition of the Ca^{2+}

ATPase. Ghosts sealed in vanadate-free medium subsequently incubated in vanadate containing medium showed a time dependent inhibition of Ca^{2+} efflux. Ghosts sealed in the presence of arterenol, that were then incubated in vanadate containing medium, did not show this inhibition of Ca^{2+} efflux. These results suggested that the vanadate was acting on the cytoplasmic face of the membrane (Rossi et al 1981).

The kinetics of uptake of vanadate incubated with rat heart cells suggested that binding to the surface was not occuring (Werdan et al 1980). Finally the negative charge of the cell surface is likely to repel the negatively charged vanadate ion.

The mechanism of uptake in erythrocytes has been proposed to be through the anion exchange mechanism (Cantley et al 1978a). Vanadate and phosphate competed for anion exchange sites. SITS (dinitrostilbene disulphonate) inhibited the initial vanadate uptake in erythrocytes (Cantley et al 1978a), and DIDS (4,4' diisothiocyanostilbene 2,2' disulphonic acid) inhibited vanadate-induced enhancement of contraction and subsequent relaxation of Guinea pig taenia coli muscle (Ueda and Urakawa 1983). Both of these, SITS and DIDS, are known inhibitors of anion exchange.

In rat heart muscle cells and rat fibroblastoid heart cells, there was no competition between vanadate and phosphate (Werdan et al 1980). However there was competition for the carrier system when different non-radioactive vanadium-containing compounds were incubated with these cells (Werdan et al 1980).

In Neurospora crassa vanadate was taken up by cells in phosphate deficient, alkaline medium, in which the high affinity phosphate transport system II was derepressed. In this case

growth was inhibited. Phosphate and vanadate were competitive inhibitors of this system (Bowman 1983). Mutants deficient in the high affinity phosphate transport system were able to grow on vanadate containing medium (Bowman et al 1983).

Brown and Gordon (1984) reported that there was an 2-3 fold increase in $pp60^{src}$ activity within intact RSV-transformed chick embryo fibroblasts. This was due to the increased phosphorylation of the protein kinase itself. Addition of vanadate to transformed cell lysates had no effect on the kinase activity. This would indicate that uptake of vanadate was essential for its action in this system.

b).Internal concentration of vanadate

Investigations of the effects of vanadate on a variety of functions of intact cells have shown that concentrations which inhibit enzymes essential for these functions in vitro do not affect these systems in intact cells. This was interpreted at the time to be due to poor entry of vanadate into the cells. It was initially thought that intact cells were relatively impermeable to vanadate (Kobayashi et al 1978). This is unlikely because Cantley et al (1978a) reported high ^{48}V-vanadium activity associated with the inside of the erythrocyte (see above). It is more likely that intracellular vanadate is reduced to the less active vanadyl 4+ form (Cantley et al 1978a; Cantley and Aisen 1979). Thus the internal vanadate concentrations would be much lower than the concentrations outside the cells.

Physiological levels of vanadium have been reported to be around $1x10^{-7}$ M - $1x10^{-6}$ M (Underwood 1977; Cantley et al 1978a).

It is likely that levels of vanadate are much lower since much of the tissue vanadate is believed to be reduced. Consideration of the electrode potentials (see Table 1) and the various main redox systems of the cell NADH $\longrightarrow$ NAD, FADH $\longrightarrow$ FAD, suggests that reduction of vanadate to the VO^{2+} (4+) state should occur. Glutathione is known to reduce vanadate (Macara et al 1980). The reduced vanadium binds to proteins such as haemoglobin in the red blood cell (Cantley et al 1978a) or transferrin and ferritin (Sabbioni and Marafante 1981). Vanadium $^{4+}$ is less effective at inhibiting many of the systems described in the introduction. For example ascorbic acid has been reported to overcome partially Na/K ATPase inhibition by vanadate in vitro (Grantham and Glynn 1979) and reverse experimentally induced vanadium poisoning in vivo (Mitchell and Floyd 1954). Addition of arterenol, which reduces the vanadate and forms a complex with the vanadyl ion, can reverse some of the effects of vanadate (Josephson and Cantley 1977). Although as discussed above there are some cases where the reduced V+4 ion has an inhibitory effect on enzyme systems (see Table 2).

Taking into account the physiological environment and chemical nature of vanadium Rubinson (1981) has predicted that extracellular vanadium will be in the form of vanadate and intracellular vanadium will be predominantly in the vanadyl 4+ form.

My results from the work using radioactive vanadate indicate that there was some uptake of the isotope into the cells. In cell-free systems vanadate is known to have various effects on the enzymes concerned with DNA and protein synthesis (see introduction). Results I obtained investigating both systems

would indicate that at the concentrations that were inhibitory in cell-free systems there was little apparent effect on the cell monolayers for a period of 12 hours (see Fig 11b). If much of the cell-associated vanadium in my experiments was not in the form of the vanadate ion, but had been reduced to the 4+ vanadyl ion, the actual concentration of vanadate present would have been considerably lower than that initially added. This could account for the lack of inhibition of protein and DNA synthesis when vanadate was added to the intact cells.

Gordon et al (1983) used intact cells and found that vanadate ($1x10^{-6}$ M-$1x10^{-4}$ M) did not alter the overall incorporation of ^{32}P or ^{35}S methionine into cellular proteins. Seglen and Gordon (1981) reported that $1x10^{-2}$M vanadate inhibited protein degradation in isolated rat hepatocytes but did not affect cell viability or protein synthesis over the 2 hour time course although the cells became rounded and highly blebbed. No inhibition of the sodium pump activity was observed when $5X10^{-5}$M vanadate was added to intact 3T3 cells (Smith 1983). Again reduction of cell-associated vanadate to the vanadyl 4+ form could account for the lack of inhibition of protein synthesis or sodium pump. This would also explain why the sodium pump activity was inhibited, in intact erythrocytes, at vanadate concentrations 3 orders of magnitude greater than that required to inhibit the isolated enzyme (Cantley et al 1978a; Beage et al 1980; and see above). Similarly the motility of intact sperm was inhibited only at concentrations 2 orders of magnitude greater than concentrations required to inhibit demembranated sperm (Kobayashi et al 1978).

2. Mechanisms Of Vanadate Inhibition Of Spreading And Aggregation

In view of the low effective internal concentrations which seem to be achieved, vanadate may inhibit cell aggregation and cell spreading by exerting relatively specific effects on a small number of enzymes which are particularly sensitive to the presumed low concentrations of vanadate found within the cell.

The identity of such potential targets is presently open to speculation. It is not even established that the inhibition is via an intracellular mechanism. The results obtained from my experiments using radioactive vanadate suggest the possibility that there may be adsorption of the vanadium species present to the substrate and/or the cell surface. There is no evidence to rule out the possibility that this surface adsorption is responsible for some of the effects of vanadate on intact cells. There is evidence that the VO^{2+} (vanadium 4+) ion does adsorb to the surface fatty acids (Chasteen and Hanna 1972; Stilbs et al 1977). However there are no reports in the literature concerning the adsorption of the vanadate ion, the form that is most likely to occur extracellularly (Rubinson 1981). The effect of vanadate on adhesion may be explicable in terms of the alteration of the electrostatic forces, thus increasing repulsion between adjacent cell surfaces (Curtis 1973). Such data about vanadate adsorption suggests that the sign of charge of the ion would be the opposite to that which would diminish adhesion (Chasteen and Hanna 1972; Stilbs et al 1977). Neither is it clear whether aggregation and spreading are inhibited via a common target or by dissimilar mechanisms. Some possible targets can probably be discounted. For aggregation the use of ouabain has indicated that it is unlikely that the Na/K ATPase is involved (Edwards 1981). cAMP

has been shown to enhance aggregation and therefore the stimulation of adenylate cyclase is unlikely to be involved in the inhibition seen with vanadate (Edwards unpublished observations). My work has shown that both of these systems are unlikely to be involved in spreading. Myosin seems an unlikely target in view of the slow rate of onset of inhibition in vitro (Magid and Goodno 1982). In the case of aggregation a dynein-like species could be involved in the inhibition by vanadate. This inhibition is paralled by a range of microtubule-depolymerising agents (Edwards 1981). However, in spreading assays colchicine failed to reproduce the effects of vanadate (see below).

A possible mechanism which could apply to inhibition both of aggregation and spreading is increase in tyrosine phosphorylation, either by stimulation of tyrosine kinases or by inhibition of tyrosine phosphatases as will be discussed below. There is some circumstantial evidence for the increase in tyrosine phosphorylation from my work as well as evidence in the literature:

1. Both the inhibition of aggregation and the inhibition of spreading parallel effects of viral transformation.

Polyoma transformed and Rous sarcoma virus transformed BHK cells aggregate less than the untransformed parental C13 cells (Edwards et al 1979). Transformed cell lines have many morphological and behavioural differences from their normal counterparts and frequently are more rounded than non-transformed cells (Gordon et al 1983; Weinberg 1984). These effects resemble those of vanadate where aggregation is inhibited (Edwards 1981)

and cells are rounded (see results above).

2. Vanadate has effects on both a) isolated enzymes and b) intact cells which could result in increased phosphotyrosine levels.

a). Vanadate stimulated tyrosine phosphorylation of isolated membrane proteins at concentrations of $1x10^{-6}M$ -$1x10^{-4}M$ (Ushiro and Cohen 80; Earp et al 83; Tamura et al 83). At concentrations of $5x10^{-5}M$ vanadate inhibited the phosphotyrosine histone dephosphorylation of isolated plasma membrane of human astrocytoma (Leis and Kaplan 1982).

Vanadate has been shown to have some growth factor-like effects in some cells(Carpenter 1981), although no such effects were found during this work. It has also been shown to stimulate the tyrosine phosphorylation of the insulin receptor in vitro (Tamura et al 1983). A number of growth factors may exert their effects through phosphorylation of tyrosine. The best known example is that of EGF where increased tyrosine phosphorylation was seen when EGF bound to specific surface receptors, stimulating cells to divide. One of the most rapid responses to this was the activation of an intracellar tyrosine protein kinase (Ushiro and Cohen 1980). This is now known to be part of the receptor molecule (Hunter 1982). PDGF bound to specific cell receptors, distinct from those for EGF, and this was accompanied by an increased cellular tyrosine protein kinase activity (Ek et al 1982). Insulin receptors were also associated with a tyrosine specific protein kinase (Zick et al 1983). Vanadate has also been demonstrated to potentiate the mitotic action of some growth factors on some cells in culture (Carpenter 1981; Smith 1983 and see below).

b).Vanadate has been shown to have certain actions similar to that of the polypeptide growth factors. It has been shown to stimulate Na^+/H^+ exchange in A431 cells, leading to an elevation of cytoplasmic pH by a mechanism similar to EGF (Cassel et al 1984). Vanadate has been shown to act - synergistically with EGF (Carpenter 1981) in quiescent human fibroblasts, to stimulate DNA synthesis. Smith (1983) found that vanadate stimulated DNA synthesis in quiescent 3T3 and 3T6 cells in serum free media, and acted synergistically with 1%-4% serum. There was a large co-operative effect with vanadate and insulin in serum free medium in a manner thought to be due to the stimulation of the cells rather than a chemical modification of the insulin (Smith 1983). $2x10^{-6}$M - $1x10^{-5}$M vanadate has been shown to potentiate the mitotic action of insulin on cultured mouse mammary gland (Hori and Oka 1980). Colchicine and other anti-microtubule agents had effects on DNA synthesis similar to those of vanadate and acted synergistically with some growth factors (Friedkin et al 1979). Taxol almost totally prevented the stimulation of DNA synthesis by vanadate (Smith 1983). Friedkin and Crawford (1983) have proposed that cytoplasmic microtubules must remain disaggregated during the prereplicative G1 phase of the cell cycle for the synergy between colchicine and growth factors to be observed. Friedkin and Rozengurt (1980) have proposed that the receptor-mitogen complex is internalised and that in the presence of disaggregated microtubules the complex may remain active within endocytotic vesicles. The lysosomal degredation of these vesicles may be retarded in the presence of dissaggregated microtubules. In view of the reports of synergy between vanadate and growth factors (Carpenter 1981; Smith 1983) it could be proposed that the association between microtubules

and intermediate filaments is also important in the prolongation of the life of the receptor-mitogen complexes within the cell.

3. Vanadate ($1x10^{-6}$M - $1x10^{-4}$M) has been shown to stimulate the protein kinase activity of $pp60^{src}$ in RSV transformed chick embryo fibroblasts and NY-68 cells. These effects were reversible and vanadate did not alter the overall protein phosphorylation or protein synthesis (Gordon et al 1983). Purified $pp60^{src}$ protein isolated from cells after incubation in the presence of $1x10^{-5}$ M vanadate showed an isoelectric variant with increased phosphorylation of the protein itself at the aminoterminal (Brown and Gordon 1984; Collett et al 1984). This isoelectric variant had increased src kinase specific activity (Collett et al 1984). It has been proposed that the $pp60^{src}$ activity could be regulated by a vanadium-sensitive phosphotase activity (Collett et al 1984). A search for electrophoretic charge variants of proteins from BHK cells treated with vanadate might have been valuable.

4. An apparent synergy between vanadate and transformation

My results have shown that transformed cells prespread on fibronectin are more sensitive to the rounding effects of vanadate than their untransformed counterparts. Py and ASV cells rounded within 2 hours of the addition of vanadate whereas C13 cells remained spread for the duration of the assay (see Figures 18,19 and 20 and Plates 2,3 and 4).

The evidence discussed above suggests that vanadate may alter the cellular levels of protein phosphorylation, especially that of phosphotyrosine. There are several possible targets of vanadate action in this respect which are considered below.

Possible Targets In The Inhibition Of Spreading And Aggregation

Several possible mechanisms for the inhibition by vanadate of spreading and aggregation via protein phosphorylation are considered below. These include the alteration of phosphorylation of proteins associated with adhesion plaques either through the stimulation of kinases that specifically phosphorylate tyrosine or by inhibition of phosphotyrosine phosphatases. Alternatively vanadate could affect the ras protein in an analogous manner to the activating mutation thus blocking the GTPase activity of the protein (see below).

Some transforming gene products, tyrosine protein kinases, are known to phosphorylate proteins of the cytoskeleton, vinculin, and to a lesser extent filamin and vimentin (Sefton et al 1981). Vinculin is concentrated at sites of actin-membrane interaction and cell-substratum contact. It has been suggested that it is involved in attachment of microfilaments to the inner face of the membrane (Geiger 1979; Burridge and Feramisco 1980). Talin, a 215Kd protein has also been found to localise at adhesion plaques (Burridge and Connell 1983) and to interact with vinculin (Burridge and Mangeat 1984). It is a phosphoprotein, but it is not yet known if it contains phosphotyrosine (Burridge and Connell 1983). Ball and Singer (1981) have shown the rapid dissociation of the alignment of microtubule and IF displays upon transformation by a ts-RSV mutant in cultured NRK fibroblasts. They speculated that the link between these two filamentous systems could be a possible substrate for the $pp60^{src}$ (Ball and Singer 1981).

In many transformed cells focal contacts are altered in

organisation and the microfilaments bundles are disrupted (Hanafusa 1977; Shriver and Rohrschneider 1981). In RSV transformed cells the pp60^{src} (transforming gene product) is found in close association with the altered distributions of vinculin and alpha actinin (Shriver and Rohrschneider 1981; See Hunter 1982 and Hynes 1982 for more detailed discussion). As mentioned above vanadate has been shown to stimulate pp60^{src} protein kinase in RSV transformed cells (Gordon et al 1983,1984).

There is a problem with the idea that tyrosine phosphorylation of vinculin causes the morphological changes, because on transformation levels of phosphorylation of vinculin reach a stoichiometry of only 0.01 phosphotyrosine per vinculin molecule (Hynes 1982). This level of phosphorylation could be effective if there is a rapid turnover of phosphate (Sefton et al 1980) or if phosphorylation of a small fraction of the total vinculin is crucial (Hynes 1982). Reverse transformation, by the addition of cAMP, causes a reversal of many of the morphological transformed characteristics, this reversal includes the flattening of cells. There is some phosphorylation and dephosphorylation of various cytoskeletal proteins during this process (Lockwood et al 1981). This phosphorylation/ dephosphorylation may be important in the control of expression of normal/transformed morphologies.

Some phosphotyrosine-specific phosphatases have been described (Foulkes 1983) and although their functions have not been elucidated, it is thought that they may regulate tyrosine phosphorylation. Protein phosphorylation in general is thought to be a major regulatory feature of protein function (Foulkes 1983). Vanadate ($5x10^{-5}$ M) has been shown to inhibit

phosphotyrosine dephosphorylation in isolated membranes of human astrocytoma (Leis and Kaplan 1982; Foulkes 1983).

Another aspect of protein phosphorylation relevant to this study is that concerning the irregular appearance of the nuclei in vanadate treated cells (Plates 15 and 16). The proteins of the nuclear lamina become highly phosphorylated during prophase prior to the breakdown of the nuclear envelope (Gerace and Blobel 1980). Vanadate could affect the state of phosphorylation of these lamina proteins and thus alter the nuclear morphology. Alternatively the changes in nuclear morphology might be a consequence of changes in intermediate filament display since intermediate filament proteins are thought to be closely associated with the nuclear membrane (Eckert et al 1982; 1983).

Alternatively vanadate may act in a manner similar to a transforming gene product without the involvement of tyrosine specific protein phosphorylation. Ras-proto oncogene products are found localised at the inner face of the plasma membrane and are thought to be involved in transducing signals from growth factors to other parts of the cell (Newbold 1984). The ras proteins are able to bind GDP and GTP with high affinity. The normal product has a GTPase activity whereas in the activated ras proteins this activity is impaired. In addition to the effects on growth, these activated proteins are able to induce morphological transformation (Newbold 1984). The mode of action of the activated ras protein may be analogous to the binding of the G-protein to adenylate cyclase resulting in the activation of the enzyme. The impaired GTPase activity in the ras p21 may be equivalent to the permanent stimulation of the adenylate cyclase in a manner similar to that of vanadate. In view of the sequence

homology between the ras gene protein and adenylate cyclase G-protein (McGrath et al 1984) vanadate may bind to the ras proteins, inhibiting their GTPase activity and thus bringing about morphological transformation (or specifically rounding). EGF and insulin have been shown to enhance the GDP and GTP binding to ras proteins in isolated membranes (Kamata and Feramisco 1984). Although the EGF receptor probably does not rely on these G-like proteins for signal transduction it is becoming increasingly likely that the transforming ras proteins are involved in the disruption of normal growth factor controls on cell proliferation (Newbold 1984).

Possible Mechanisms Of Inhibition Of Cell Aggregation Involving Cytoskeletal Elements

Colchicine and vinblastine inhibit fibroblast aggregation (Waddell et al 1973). Vanadate also inhibits cell aggregation in a way closely parallel to that of colchicine (Edwards 1981). I have shown that colchicine does not inhibit the spreading of fibroblasts on fibronectin.

Colchicine and other microtubule depolymerising agents cause a disruption of the microtubule networks throughout the cytoplasm and also induce perinuclear whorls of intermediate filament proteins (see introduction). Vanadate ions induce the intermediate filament coiling, leaving microtubules intact (Wang and Choppin 81). The vanadate concentrations effective at producing the coiling of IF are the same as those that cause the inhibition of aggregation (and spreading). Vanadate inhibited the dynein ATPase of intact sperm at concentrations of 1×10^{-4} M

(Kobayashi et al 1978). Thus if the action of cytoplasmic microtubules is dependent on the action of a dynein-like protein this could be a candidate for the inhibition of aggregation seen with both vanadate and colchicine.

There are several possible ways in which the inhibition of a dynein-like species could inhibit cell aggregation. It could be involved in the maintenance of If displays within the cell. Redistribution of the filaments following inhibition of a dynein-like species could account for the inhibition of aggregation. Alternatively a dynein-like species could be involved in the secretory mechanism within the cell. Inhibition of the secretory pathways could affect the secretion of proteins to the external medium or the delivery and insertion of new plasma membrane components. Lastly, microtubules may be involved in the positioning of components in the plane of the membrane (Waddell et al 1974). There is some recent evidence for such microtubule-dependence of the positioning of acetylcholine receptors (Connolly 1984)

The inhibition of aggregation by vanadate could involve the inhibition of a dynein-like ATPase involved in the microtubule-dependent deployment of intermediate filaments (Edwards 1981). It was suggested that intermediate filaments may attach to the inner face of the membrane (Edwards and Dysart 1980) and that such linkages might be necessary for the stability of the adhesions formed between suspended cells (Edwards 1981), by analogy with the involvement of keratin IF in desmosomes. Recent findings that desmin and vimentin are present in desmosome-like structures in muscle and meningiomal cells (Kartenbeck et al 1983; 1984) are consistent with this hypothesis.

Dynein-like enzymes may be involved in the secretory process within the cell and thus inhibit the secretion of molecules to the external surface of the cell or the delivery and insertion of plasma membrane components. Aggregation of freshly trypsinised cells may depend on secretion of surface components from an intracellular pool (Edwards et al 1979). Colchicine has been reported to inhibit the secretion of plasma proteins from rat hepatocytes within 2 minutes of administration, the effect lasted only 3 hours (Redman et al 1975). There was no apparent effect on the transport of secretory proteins in the rough or smooth ER, but there was an accumulation of golgi derived secretory bodies (Redman et al 1975). Similarly in rat parotid glands, colchicine inhibited secretion of de novo synthesised amylase, but had no effect on amylase already present within the cells. Again there were alterations in the golgi body (Patzelt et al 1977). Busson-Mabillot et al (1982) concluded that colchicine exerted an effect at the time of secretory granule formation, possibly making them unable to discharge their contents. Colchicine treatment had no effect on preformed granules in rat lacrimal gland cells. The transfer of proteins from the rough ER to the golgi was supressed and it was concluded that the microtubule system was not indispensable for transport of proteins from the rough ER to the golgi, but that microtubules might facilitate this process (Busson-Mabillot et al 1982). Mts were implicated in the function of the golgi body in plasma protein processing for the sustained secretion of insulin from beta cells (Maurice et al 1983), although they were not required for immediate release of the hormone (Boyd et al 1982). A close association between secretory vesicles and IF was reported in gonadotrophic cells of the lizard pituitary gland (Forbes and

Dent 1974). These authors suggested that intermediate filaments were involved in secretion.

My results showed that over a 4 hour period there was no apparent effect of colchicine or vanadate on secretion. The lack of inhibition within this time course is consistent with the evidence discussed above (Figure 25). The assay may have been terminated before any affect of the inhibitors, on secretory granules formed in their presence could be detected. Coated vesicles have been shown to be attached to microtubules. MAP 2 or dynein have been proposed as candidates for forming bridges between the coated vesicles and microtubules (Imhof et al 1982). The work described above would not have detected the inhibition of insertion of new plasma membrane proteins. If there was some inhibition of this intracellular transport the surface properties of the cell may have been altered and this could account for the inhibition of cell aggregation.

CONCLUSION

Vanadate has proved to be a very useful tool for the enzymologist. It has been used to inhibit a number of isolated enzymes in an attempt to understand their mechanisms of action. For the cell biologist however, its effects on intact cells are not yet sufficiently well understood for its actions to be directly interpreted in terms of specific mechansims.

My work suggests the possibility that some of its effects, especially the reversible inhibition of spreading and of cell aggregation may be mediated through one or a small number of highly sensitive enzymes. If these could be identified there would be much scope for its use in IF biology and other aspects of cell biology. A possible avenue of research, which could lead to the identification of such key enzymes is the investigation into levels of phosphorylation of specific proteins isolated from intact cells treated with vanadate.

Synergistic effects of vanadate and some polypeptide growth factors in intact cells have already been discussed in relation to DNA synthesis (see introduction and discussion). Another promising possibility for future work would be to look for synergy between vanadate and growth factors with regard to morphological effects on cells.

FOOTNOTE

Since the completion of this thesis a paper by Klarlund (1985) has confirmed some of the work reported here as well as adding further evidence to some of the points discussed above.

$1x10^{-3}$ M vanadate added to normal cells in culture medium produced cell rounding within 3-4 hours. At this concentration cell death was observed within 6-12 hours. At concentrations of $3.75x10^{-5}$ M vanadate cultures were maintained for up to one month. At $5x10^{-5}$ M vanadate survival was erratic. The effects of vanadate at these concentrations are in accordance with results reported here for the growth experiments (see figure 1 and table 4). Cells were refractile and some of the attached cells had a needle-like morphology (compare with plate 1 of this work). The effects of vanadate were dose dependant and readily reversed by washing to remove the ion from the culture medium. Reversal towards a control type morphology was apparent after 6 hours and complete within 24 hours. In my experiments reversal of vanadate action was observed within 2-3 hours (see figure 18). Differences in the time scale of the reversal of vanadate action may be due to use of HH as the incubating medium in my experiments compared to serum containing medium in the above paper.

The induction of transformation by vanadate (see discussion above page 92) was also observed by Klarlund who used four criteria to describe this effect; 1) generation of a highly refractile morphology, 2) decreased density-dependant growth inhibition, 3) increased uptake of 2-deoxyglucose and 4) growth in the absence of solid support. These effects were dose dependant and reversible upon removal of the vanadate ion.

In addition it was reported that vanadate inhibited phosphatases that removed phosphate groups from phosphotyrosine in cell free systems. Addition of the ion to intact cells caused a 40 fold increase in cellular protein levels of phosphotyrosine compared to a 5 to 10 fold increase in transformed cells (see discussion above from page 92). No change in the phosphorylation of the proteins could be detected in 1D gels. 1D gels, in this thesis, were also unsuccessful in detecting any possible changes in phosphorylation of insoluble cellular protein (see plate 17).

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