THE DEVELOPMENT AND CHARACTERIZATION OF TOLERANCE, ADDICTION, AND WITHDRAWAL BY NON-NERVOUS CELLS IN CULTURE

By

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Dedicated to my loving and understanding wife, Barbara, and to my children, Steven and Jennifer, who never cease to give purpose to my life.

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LIST OF ABBREVIATIONS

ACh	-	acetylcholine
AIB	-	α-aminoisobutyric acid
CHO-K	-	Chinese hamster ovary cells, strain K
CNS	-	central nervous system
CPM	-	counts per minute
CPZ	-	chlorpromazine
DA	-	dopamine
5-HT	-	5-hydroxy tryptamine (serotonin)
MDB	-	milk dilution bottle
MS	-	morphine sulfate
PEA	-	phenethylalcohol
PGE_1	-	prostaglandin E_1
PZ	-	promazine
RP	-	relative plating
RPM	-	revolutions per minute
S_3-HeLa	-	HeLa cells, strain S_3
Tris	-	2-amino-2-(hydroxyethyl)-1,3-propanediol
V79-CHL	-	Chinese hamster lung cells, strain V79

CHAPTER I

INTRODUCTION

This study was undertaken to develop and characterize a line of cells which can be used as a model to study tolerance, addiction, and withdrawal in cultured cells. To establish a common foundation the following will be reviewed: history of narcotics, narcotic classification and structure, pharmacology, theories for tolerance and dependence, suitability of cultured cells, opiate receptors, enkephalins, cAMP and adenylcyclase, neurotransmitters, and specific assay techniques (plating and time lapse cinematography).

History of Narcotic Drugs

Narcotic drugs have been used by society perhaps as long as there has been a society, with recorded description of cultivation and preparation of opium occurring around 7000 B.C. (1). Narcotics are used medicinally for their antinociceptive (analgesic) and sedative properties but these agents have serious side effects including tolerance and dependence. Tolerance is defined as a decreased response to the same concentration of drug with repeated administration, or conversely, as a requirement for increased drug dosage to provide the same response. Dependence refers to the altered physiological state brought about by continued administration of the drug over a long period of time. Continuation of the drug is required to prevent a set of responses referred

to as the abstinence syndrome (withdrawal). In addition to the physical dependence which develops, a psychological dependence (habituation) is also seen. The abstinence syndrome produces a variety of symptoms, differing in time of onset and degree of severity with the individual and the extent of the dependence. These symptoms include: nervousness, frequent yawning, profuse sweating, runny eyes and nose, dilated pupils, severe muscle twitching, painful cramps (back, leg, and abdominal), vomiting, diarrhea, loss of appetite, insomnia, increase in respiration, increase in blood pressure (systolic and diastolic), increase in body temperature, increase in blood sugar, and an increase in the basal metabolic rate (2). The severity of these symptoms is used to measure the degree of dependence (3-5).

Classification and Structure

Foldes, Swerdlow, and Siker (6) classify narcotic analgesics into three groups. These are:

a) natural alkaloids present in opium; includes: morphine, codeine, papaverine, laudanosine, laudanine, papaveraldine, narcotine, narceine, cotarnine, and narcotoline,

b) semi-synthetic compounds obtained by modification of morphine or codeine; includes: ethyl morphine•HCl, heterocodeine, heroin, dihydromorphine•HCl, dihydrocodeine bitartarate, hydromorphone•HCl, desomorphine•HBr, oxymorphone•HCl and others,

c) synthetic; structural resemblance to whole or part of morphine molecules; includes:

   1) Morphinan derivatives: levorphanol, dextrorphan, RO4-0288

(1,3 hydroxy-N-phenacylmorphinan•HCl), phenazocine•HBr,

2) Methadone derivatives: methadone•HCl, dipipanone, phenadoxone, isomethadone•HCl, dextromoramid,

3) Meperidine derivatives: meperidine, alphaprodine, ketobemidone, anileridine,

4) Miscellaneous: 4-anilinopiperidine (Fentanyl, R4263), 6-methylene dihydro desoxymorphine, diphenoxylate, etorphine.

These compounds vary in potency from 1/10 to 10,000X (codeine, etorphine, respectively) the potency of morphine. The structures of the narcotic agonists used in this study are shown in Table I.

The narcotic antagonists, which also resemble morphine structurally, oppose the action of morphine and can precipitate the abstinence syndrome. The most common antagonists are shown in Table II.

Pharmacological Properties

Narcotic agonists have a number of pharmacological effects. Analgesia is the most outstanding effect in man (6). Other effects include sedation (decreased sensitivity to disagreeable situations) (7), psychological euphoria (7), excitation (8), nausea (9), vomiting (9), myosis (8, 10), bradycardia (11, 12), increase in cerebrospinal fluid pressure (13), alteration of electroencephalographic patterns (14), histamine release (15), depression of respiration rate (16), depression of tidal volume (16), elevation of pCO_2 (17), alteration of breathing pattern (18), depression of the cough reflex (19), increase in biliary pressure (8), constipation (8), and a decrease in the basal metabolic rate (12). Tolerance to the depressant effects in the central nervous system (CNS) occurs, but not to the excititory

TABLE I

NARCOTIC AGONISTS

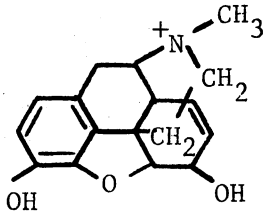
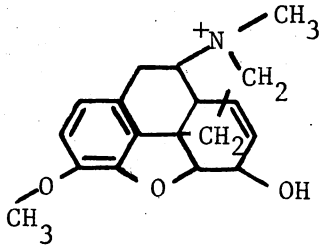
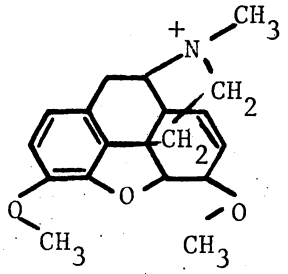
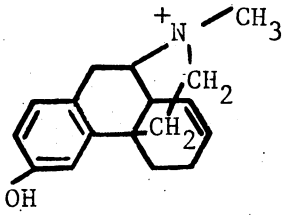
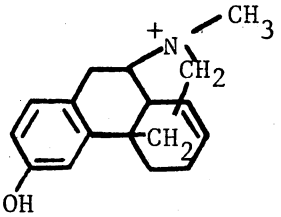
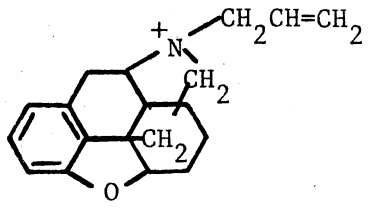
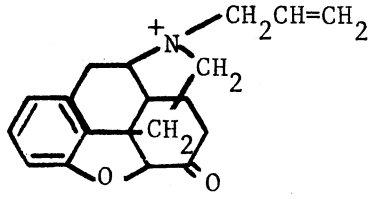
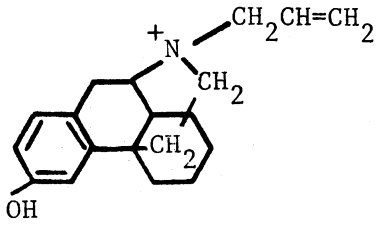
Name	Relative Potency (Morphine = 100)	Structure
Morphine	100	
Codeine	10-15	
Heroin	200-300	
Levorphanol (L-isomer)	330-500	
Dextrorphan (D-isomer)	0	

TABLE II

NARCOTIC ANTAGONISTS

Name	Relative Potency (Nallorphine = 100)	Structure
Nallorphine	100	
Nalloxone	2000-5000	
Levallorphan	500	

effects (20). Although varied, none of the pharamcological effects are believed responsible for tolerance and/or dependence.

Theories for Tolerance and/or Dependence

Several theories for tolerance and dependence have been proposed. Sharma et al. (21) find two types of adenyl cyclase in brain, one sensitive to morphine and the other not. They propose a compensatory shift in enzyme synthesis, degradation, or activity which restores the cyclic AMP levels depressed by morphine. Collier et al. (22) also propose cAMP homeostasis is involved in tolerance and dependence.

Kosterlitz and Hughes (23) propose that opiate-enkephalin interactions are responsible for tolerance and dependence. They propose that enkephalin, an endogenous peptide with morphine-like action acts as an inhibitory neurotransmitter in regulating brain pathways. Exogenous opiates remove control of these pathways from the enkephalin and gives it to the exogenous opiates. Negative feedback from the inhibited pathways reduces the endogenous enkephalin so that endogenous enkephalin levels become insufficient in the absence of exogenous opiates (dependence). Tachyphylaxis (accelerated synthesis of neurotransmitters) on depressed pathways is postulated as responsible for tolerance.

Vander Wende and Spoerlein (24) propose that an increase in the synthesis of dopamine which is a natural opiate antagonist is responsible for tolerance. As dopamine levels increase, additional morphine is required for an effect (tolerance). Presence of morphine is required to prevent overactivity caused by the high dopamine levels (dependence). Severs and Deneau (25) and Jaffe and Sharpless (26)

attribute tolerance and dependence to a latent hyperactivity of CNS synapses (dopamine receptor hypersensitivity).

Kaufman et al. (27), Goldstein and Goldstein (28, 29) and Shuster (30) propose that feedback control mechanisms responsible for maintaining a dynamic, neurotransmitter balance are responsible for tolerance and addiction. They propose homeostatic mechanisms responsible for restoring the balance after morphine perturbation are responsible for tolerance. They feel that this restoration is brought about by increased neurotransmitter synthesis, so that when opiates are removed, the homeostasis is again perturbed.

Siegal (31) proposes

> . . . that the direct analgesic effect of morphine becomes attenuated over the course of successive administration of the narcotic by a conditioned, compensatory, hyperalgesic response elicited by the administration procedure, the net result being analgesic tolerance (p. 498).

I.e. tolerance is learned. He supports this with experiments in rats in which environmental cues are required for the development of tolerance and found that a placebo administered with the appropriate cues could replace morphine in dependent rats.

Use of Cell Culture

In a review on the biochemistry of addiction, Dole (32) states

> . . . the biochemical determinants of addiction are more likely to be found in events close to the primary cellular actions of narcotic drugs than in the relatively nonspecific discharges of remote neurons . . . The common denominator of these phenomenon (tolerance and physical dependence) is the narcotic drug-cell interaction (pp. 832-833).

He also concludes that "tolerance is cellular (p. 829)". In view of this and the complexities involved in studying whole animal systems, cell culture becomes an ideal model system for assessing the changes involved in tolerance and dependence.

Cell culture has been used extensively to ascertain the effects of various agents on cell parameters. In comparison with more complex test systems, cell culture offers several advantages including: free access of the drug to the cells, lack of changes in drug concentration due to excretion methods, homogeneity of the cell population and a strictly controlled and easily variable environment for the cells (33). In addition to these advantages, Pomerat and Leake (34) add simplicity of equipment and methodology and the possibility of recording results in a dynamic time frame. Disadvantages in cell culture systems include: functional changes not appearing as structural changes may be missed, loss of interaction between cell types and cell systems, and drugs that might be metabolized to effective forms *in vivo* might be missed *in vitro* (35).

Effects of Non-Narcotic Drugs on Cells in Culture

The effects of non-narcotic drugs on various cells in culture have been studied extensively. Using U.V. irradiated mouse L cells, Damon and Rauth (36) found caffeine decreased colony forming ability. Thayer *et al.* (37) found chronic exposure to low caffeine doses did not cause chromosome breaks in HeLa in contrast to the breaks found with acute exposure to high doses. Dybing (38) showed that chloropromazine [(10-(dimethylaminopropyl)-2-chlorophenylthiazine•HCl), CPZ] and

SKF-525A (diethylaminoethyldiphenylvalerate) inhibit uptake and incorporation of alanine and α-aminoisobutyric acid (AIB, a non-metabolizable amino acid) by hepatoma cells in culture. Using human skin epithelial cells (HE), Dybing (39) finds CPZ inhibits alanine and AIB uptake and incorporation but does not inhibit phenylalanine or cycloleucine. He also shows actinomycin D stimulates thymidine uptake, inhibits hypoxanthine uptake, and has no effect on phenylalanine or cycloleucine uptake. Roubein et al. (40) studied the effects of CPZ and mescaline (both cytotoxic agents) on cerebellum and fibroblasts, finding CPZ more toxic than mescaline with cerebellum more sensitive than fibroblasts. Using enzyme leakage (lactate dehydrogenase, malate dehydrogenase, and aspartate aminotransferase) as a measure of toxicity, Dujovne and Zimmerman (41) found CPZ to be more toxic than promazine (10-(dimethylaminopropyl)-(phenothiazine•HCl), PZ) to Chang liver cells (human). Pomerat, Finerty, and Perry (42) compared CPZ to thorazine (commercial preparation of CPZ, ascorbic acid, sodium bisulfite, sodium chloride, and sodium sulfite; used as a tranquilizer, antiemetic, sedative, and potentiator) on chick embryo explants (spinal cord, heart, and spleen) finding thorazine to be more toxic than CPZ. Spinal explants were most sensitive with spleen explants least sensitive.

Schaefer (43) found that 5,5 diphenylhydantoin (sodium dilantin) stimulated the growth of fibroblast but not epithelial cells.

Scaife (44) used synchronized human T cells (kidney) to study the effects of barbiturates, cyclohexamide, and pederin on different points in the cell cycle. Pentabarbital blocks cell at mitosis similar to colchemid, but amylobarbital, cyclobarbital, and diethylbarbital do not. Cyclohexamid and pederin block the cells in prophase. Pomerat et al.

(45) studied barbiturate inhibition of outgrowth of chick embryo spinal cord and heart explants. Total inhibition of spinal cord was achieved at 1:6400 for sodium seconal, and 1:3200 for pentabarbital sodium and sodium amytal. Heart cells were less sensitive. Pomerat states "the convulsive, lethal, and anaesthetic doses of fourteen other barbiturate compounds was generally well correlated with *in vitro* estimates of toxicity" (p. 325).

Higgins *et al*. (46) studied the effects of phenethylalcohol (PEA) on mouse L cells. PEA, which does not affect isolated chromosomes, altered the staining and distribution of chromosomes in the cells with a loss of DNA observed. PEA decreased [^{3}H]-uridine incorporation, and activated and increased release of lysosomal enzymes.

Harrison, Kleiger, and Merigan (47) used isolated, beating chick embryo heart cells in culture to study the effects of isoproterenol, a specific beta-adrenergic stimulating agent. Isoproterenol stimulated beating in the cells. Propanolol, a beta adrenergic blocking agent could prevent the isoproterenol stimulation. Mercer and Dower (48) studied the effect of digoxin, procaine amide, and quinine sulfate on isolated beating chick heart cells. Digoxin increased the total number of beating cells and increased the percentage of arrythmic cells where procaine amide and quinine sulfate decreased the total number of beating cells but had no effect on the percentage of arrythmic cells. Quinine/digoxin combinations increased the number and proportion of arrythmic cells (greater than digoxin alone).

These studies show several important points. These include the use of cells in culture to study the toxicity of various drugs, and that drugs believed to act on neural tissue (CPZ, PZ, barbiturates)

also show effects on non-neural tissue.

Effect of Narcotics on Non-Neuronal Tissue

Toxicity of various narcotics to HeLa cells in culture has been looked at by several authors (49-51). Simon (49) compared toxicity of several narcotics in mice and HeLa cells, finding a good correlation between toxicity in mice and in HeLa cells. Simon finds an LD_{50} for HeLa cells (plating assay) of 5×10^{-4} M for morphine with dextrorphan 6X and levorphanol 10X the relative toxicity of morphine. Dextrorphan had 3-4X and levorphanol had 5-6X the relative toxicity of morphine in mice. Notebloom and Mueller (50) report 13% inhibition of growth of HeLa monolayers at 10^{-3} M morphine and no inhibition by 10^{-4} M morphine. RNA and protein synthesis in HeLa cells was inhibited equally by levallorphan and levorphanol at 1-2 mM (51).

Early evidence that cells in culture could show phenomena resembling tolerance and physical dependence came from the laboratory of Ozaki at the University of Kyoto, Japan in the early 1930's (52-56). Semura (52) showed that fibroblasts from chick embryos could become addicted to morphine and that their tolerance of morphine was gradually increased in a medium containing morphine. Sanjo (53) obtained the same results using iris epithelium. Okuda (57) found an increased tolerance to morphine by leukocytes from chickens addicted to morphine. Saito (58) observed a cross tolerance of heroin and other opium alkaloids to cultures of iris epithelium addicted to morphine. Sasaki (55) reported morphine addiction and withdrawal of primary chicken heart fibroblasts. Ten μmolal morphine had no effect on growth, 33 μmolal morphine inhibited growth, and 3.3 m molal morphine totally suppressed

growth of cells (measured as an outgrowth from the explant compared to the control). After three passages in 33 μmolal morphine, 33 μmolal morphine no longer inhibited growth. Sasaki then increased the morphine concentration to 100 μmolal morphine. This concentration initially inhibited growth, but after three passages, 100 μmolal morphine no longer decreased growth. Sasaki again increased the morphine concentration to 166 μmolal morphine, finding initial inhibition which was no longer present after three passages at 166 μmolal. Removal of morphine from these cultures after growth for nine passages in the presence of morphine (3 at 33 μmolal, 3 at 100 μmolal, and 3 at 166 μmolal) caused an abrupt decrease in growth and cellular degeneration which could be reversed for up to three passages by the addition of morphine. This can be seen in Figure 1 (from Sasaki (55), Figure 4). Heroin could substitute for morphine for reversal of withdrawal.

Heubner et al. (59) did similar studies using chick embryo leg muscle explants. Tolerance was observed as a disappearance of pathological symptoms such as fat globules and rounded cells. After 10 passages, 19/20 cultures had decreased growth rates. The growth rate of the 20th culture at 3.4×10^{-4} M morphine was greater than the control, but no decrease in growth upon withdrawal of morphine was observed. Unfortunately, all the cultures were lost at the 19th passage.

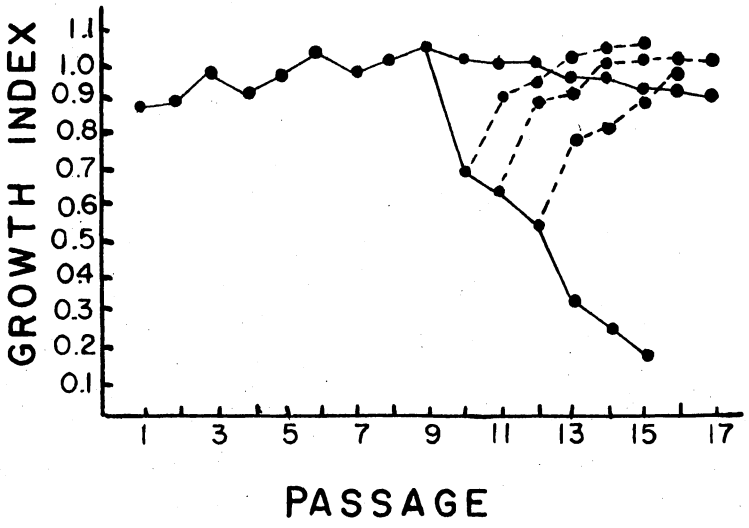
McCormick and Knikes (60) addicted rats to morphine and levorphanol (dromoran) by increasing injections of morphine or levorphanol to levels of 630 mg/kg and 210 mg/kg, respectively. They then explanted various tissues (corpus callosum, lung, skin, and omentium) and tested for tolerance and addiction. They found that tissue explants from addicted

Figure 1. Tolerance and Addiction in Chicken Heart Fibroblasts.

Morphine concentration used: Passages 1-3, $\frac{1}{30,000}$ (33 μM); 4-6, $\frac{1}{10,000}$ (100 μM); 7-17, $\frac{1}{6,000}$ (166 μ M).

Growth index is measured as $\frac{\text{explant area (experimental)}}{\text{explant area (control)}}$.

Morphine is removed from cells at passage nine (decrease in growth). Dotted lines show growth when morphine was added back to cultures from which morphine had been removed.


GROWTH INDEX
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
3
5
7
9
11
13
15
17
PASSAGE

animals thrived (as measured by outgrowth of new cells from explant) at levels of morphine that killed tissue explanted from control animals. No evidence of addiction was observed.

Corssen and Skora (61) used a human, epithelial like cervical carcinoma cell to study addiction and withdrawal. Concentrations of less than 500 μmolal morphine did not inhibit growth, but concentrations greater than 1.3 mM arrested growth with subsequent death of the cells. Withdrawal of morphine from cells grown 10-20 passages at 500 μmolal morphine results in rapid cell degeneration and cell death. Addition of morphine not later than two days after withdrawal prevented cell death.

In contrast to these observations of tolerance and addiction shown by non-neuronal cell lines in culture, Ruffin, Reed, and Finnin (62) did not observe any tolerance or physical dependence of H. Ep. 2 human epithelial (skin carcinoma) cells to morphine during a 154 day experiment using up to 500 μmolal morphine, but did observe morphine induced cytotoxicity.

Effects of Narcotics on Neuronal Tissue

Ghadirian (63) used nervous tissue explants from newborn rabbits and puppies to study the effects of morphine. Using an arbitrary scoring (+, -) system as a measure of growth, he found low concentrations of morphine (0.05-0.5 mg %) stimulated growth, intermediate concentrations (10-20 mg %) inhibited growth, and high concentrations (50 mg %) killed the cells. Cells conditioned to morphine by growth in increasing concentrations of morphine could grow at 50 mg % morphine sulfate. Withdrawal of morphine from these conditioned cells caused

cellular changes and a decrease in growth that could be reversed by the addition of morphine.

Several authors have looked at tolerance, addiction, and withdrawal in neuroblastoma and neuroblastoma hybrid lines. The neuroblastoma cultures exhibit many characteristics of differentiated neurons including marker enzymes for neurons (64-70) described by Wilson et al. (71). Neuroblastoma clones can be maintained in a dynamic state which gives them an advantage over the relatively static primary nervous tissue culture. Since primary nervous tissue has shown tolerance and addiction (60-63) and is more sensitive to morphine than non-nervous tissue (24, 34), several workers (72-76) have used these lines.

Manner et al. (72) studied short and long term effects of morphine on human neuroblastoma IMR-32. Acute exposure to morphine increased doubling time from 31 to 53 hours. Chronic exposure (30 generations) to 3×10^{-4} M morphine produced cells which could grow at 5×10^{-4} M morphine, although at a reduced rate. Removal of morphine from chronically exposed cells increased the doubling time. Chronic exposure to morphine produced a seven fold increase in cholinesterase activity and a ten fold decrease in choline-O-acetyltransferase activity. Acute exposure to morphine had no effect on these enzyme activities.

North and Martin (73) studied the effects of morphine, levorphanol, and dextrorphan on neuroblastoma and hepatoma cells. They found a decreased inhibition of growth (measured as number of cells/ml in spinner cultures) on the second administration of morphine which they interpret as tolerance development. Neuroblastoma cells were inhibited by morphine and levorphanol but not by dextrorphan whereas the hepatoma cells were inhibited by all three, with dextrorphan as potent as

levorphanol.

Traber et al. (74, 75) studied effects of morphine sulfate, levorphanol and dextrorphan on PGE_1 stimulated cAMP levels in neuroblastoma-glioma hybrids and neuroblastoma clones. They found morphine and levorphanol could antagonize the PGE_1 stimulation of cAMP where dextrorphan could not and that morphine and levorphanol antagonism of PGE_1 stimulation could be blocked by naloxone.

Klee and Streaty (76) have shown that a neuroblastoma/glioma hybrid could bind narcotic analgesics in a stereospecific manner with high affinity.

Effects of Narcotics on Other Organisms

Narcotics are inhibitory at high concentrations in other systems including E. coli (77), B. subtilis, B. megaterium, Micrococcus lysodeikticus, Diplococcus pneumoniae (49), Staphylococcus aureus (78), and Amoeba proteus (79). In Zimmerman's study on A. proteus (79), he found nalorphine antagonism of morphine inhibition, the only such demonstration in microorganisms. Cardosi and Schuel (80) used sea urchin eggs as a model to study the inhibitory effects of narcotics on secretion, showing a dose relation from 10^{-7} to 10^{-3} M morphine.

Opiate Receptors

Opiates interact stereospecifically with a "morphine receptor", both in animals and in cell systems.

Cuatrecassas (81) describes the requirement for specific binding. He states the binding is surmised to reflect 'specific' receptor interactions if it demonstrates:

a) strict structural and steric specificity;

b) saturability, which indicates a finite and limited number of binding sites;

c) tissue specificity in accord with biological target cell sensitivity;

d) high affinity, in harmony with the physiological concentration of the hormone; and

e) reversibility, which is kinetically consistent with the reversal of the physiological effects observed upon removal of the hormone from the medium.

Although the above description is for hormone/receptor interactions, these requirements should also hold true for opiate/receptor interactions. Cuatrecassas also discusses several problems, including binding to non-specific adsorptive sites (can show apparent high affinity and saturability, indicating a second site), binding to enzymes and/or non-biological materials present. Stereospecific binding, for example, has been observed for D,L isomers of tryptophan to albumin (81) and for levorphanol and dextrorphan to glass fiber filters (82). Gillette (83) and Klotz (84) also review drug protein interactions. Bush and Alvin (85) review the classical methods of determining drug-protein interactions, i.e., dialysis, ultrafiltration, and gel filtration.

Goldstein et al. (86) first described stereospecific binding of [^{3}H]-levorphanol to mouse brain homogenates. They distinguish between non-saturable and saturable binding. Non-saturable binding includes drug surrounded by osmotic particles and membrane bound drug involved in lipid/H_2O partitioning. Saturable binding can be non-specific,

i.e. interactions due to ionic bonds, hydrogen bonds, and hydrophobic forces, or can be specific, i.e. binding to a receptor which triggers the chain of events leading to the pharmacological effect. Goldstein et al. (86) use binding of [^{3}H]-levorphanol to distinguish these effects. They found that only 2% of the binding was due to stereospecific binding. Pert and Snyder (87), Simon et al. (88), and Terenius (89) all used modifications of Goldstein's procedures to demonstrate stereospecific binding to brain homogenates.

Pasterak and Snyder (90) identified a second, high affinity binding site for opiates in rat brain. They feel that these binding sites may explain the difference in agonist/antagonist interactions and the Na^+ effect. In the Na^+ effect (91) binding of antagonists is enhanced by Na^+ whereas binding of agonists is decreased by Na^+. Only Li^+ can substitute for Na^+. Pert and Snyder (91) propose a conformational change between the two types of binding sites to explain the effect. Heller and Simon (92) also propose a conformational change induced by Na^+. They find that Na^+ or prior opiate administration protects against a decrease in binding caused by addition of n-ethyl maleimide (a sulfhydryl group is believed to be essential for binding). Their model involves a change between a monomer/dimer with conformational changes in the dimeric state to allow the Na^+ effect. The monomer is not proposed to be sensitive for Na^+.

Opiate receptors are sensitive to a variety of treatments. Pasternak and Snyder (93) found the receptor is sensitive to trypsin (E.C. 3.4.4.4), chymotrypsin (E.C. 3.4.4.5), phospholipase A (E.C. 3.1.1.4), and high concentrations of phospholipase C (E.C. 3.1.4.3) and is insensitive to phospholipase D (E.C. 3.1.4.4), neuraminidase

(E.C. 3.2.1.18), RNase (E.C. 2.7.7.16) and DNase (E.C. 3.1.4.5). Agonist binding is more sensitive to trypsin and chymotrypsin than antagonist binding, with the receptor more sensitive to enzymes in the presence of Na^{+}. Wilson et al. (94) also find that iodacetamide increases binding of [^{3}H]-antagonists but decreases binding of [^{3}H]-agonists.

Pasternak et al. (95) found that Mn^{2+}, Mg^{2+}, and Ni^{2+} enhance the binding of agonists but have no effect on antagonists. Ca^{2+} and Fe^{2+} lower the binding of agonists more than antagonists. Na^{+} potentiated these effects. In addition to affecting binding, Ca^{2+} alters development of addiction and abstinence (96).

Creese and Snyder (97) find a strong correlation ($R = 0.97$, $p < 0.001$) between the pharmacological potency of the opiates and their binding to receptors in guinea pig illium.

Loh and Cho (98) propose cerebroside sulfate is an integral part of the opiate receptor. Cerebroside sulfate shows high affinity, stereospecific binding, and a high correlation between binding and pharmacologic potency. Cerebroside sulfate has the proper physical dimensions for interaction with the opiates.

Pert and Snyder (99) found in vivo administration of opiates increased in vitro binding of [^{3}H]-opiates, but stated the effect was highly dependent on conditions and preparation. They also stated "utilizing binding studies in vitro, we were unable to detect alterations in opiate receptors which are related to the development of tolerance and physical dependence" (99, p. 853). Klee and Streaty (100) find that

> . . . neither the number, nor the binding affinity, nor the specificity of narcotic receptor sites is changed in the morphine dependent (and therefore also tolerant) rat brain when compared with that of the normal animal (p. 61).

Klee and Nirenberg (76) determined the receptor concentration in a neuroblastoma glioma hybrid, finding a receptor concentration of 0.6 p mole/mg of protein which calculates to 3×10^5 receptors/cell (1 mg of protein ~ 1.2×10^6 cells). Baran et al. (101) determined opiate receptor levels in different mice strains finding genetic differences in the number of receptors (from 2.5×10^4 up to 4.5×10^5/cell depending on strain) but found no correlation to the genetic differences in analgesic response. This is in agreement with the observation of Pert, Aposhian, and Snyder (102) who found no correlation between levels of opiate receptors in different species and their response to analgesic drugs. Pert and Snyder (103) found a receptor concentration of 0.3 mM/g in rat brain, which using Klee and Nirenberg's (76) estimation of 1.2×10^6 cells/mg, calculates to 1.5×10^{10} receptors/cell. Pert and Snyder (99) find receptor concentration in mouse brain to be 25.1 p moles/mg (which calculates to 1.26×10^7 receptors/cell). Thus we can see a very large difference in receptor estimates (2.5×10^4/cell to 1.5×10^{10}/cell) with very little difference in the levels of opiates required for pharmacological action.

cAMP/Adenyl Cyclase

Since Klee and Streaty (100) find no receptor changes in morphine tolerant rats compared to control rats, they propose that tolerance and addiction are modulated by changes subsequent to the receptor. Cyclic AMP serves as a messenger subsequent to hormone-receptor interactions

(104). In order to determine if cAMP or adenyl cyclase are involved in tolerance or dependence, several authors looked at the interactions between cAMP and/or adenyl cyclase and opiates.

Chou et al. (105) found cerebral adenyl cyclase was activated by morphine. Ho, Loh, and Way (106, 107) found that administration of cAMP, dibutyryl cAMP, or phosphodiesterase inhibition antagonized morphine induced analgesia in non-tolerant and tolerant mice. They also found cAMP administration accelerated the development of tolerance and physical dependence on morphine. Cyclohexamide was able to prevent this cAMP effect. Collier and Roy (108) found that morphine inhibits PGE_1 stimulation of cAMP in rat brain homogenates, but had no effect on basal cAMP levels. Clouet and Iwatsubo (109) found that morphine has no effect on basal adenylate cyclase activity and that acute administration of morphine decreased cAMP level. Sharma, Nirenberg and Klee (121) found both basal and PGE_1 stimulated adenyl cyclase levels in neuroblastoma x glioma hybrids are inhibited by morphine.

Collier and Frances (110) find cAMP but not cGMP intensifies the abstinence syndrome. Mehta and Johnson found that the abstinence syndrome is directly related to brain cAMP levels.

Collier et al. (22) review effects of morphine, prostaglandin, and cAMP in the brain, proposing, as do Sharma et al. (21), that adenyl cyclase/cAMP homeostatic and compensatory mechanisms are involved in tolerance, dependence, and withdrawal.

Enkephalins

Terenius and Wahlstrom (111, 112) and Hughes (113) describe an endogenous substance in the brain which acts as an agonist at opiate

receptor sites. Hughes et al. (114) have sequenced these peptide factors and identified the two pentapeptide factors as methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu). Methionine enkephalin is present as residues 61-65 in β-lipotropin in man (115). Kosterlitz and Hughes (23) propose that endogenous enkephalin acts as a neurotransmitter or as a modulator of neurotransmission. Goldstein (116) found endorphin (residues 61-91 of β-lipotropin) also has agonist activity. Urca et al. (117) find that both morphine and enkephalins stimulate firing in the periaqueductal grey matter, a brain area which they feel is involved in analgesia. Simantov and Snyder (118) show that the enkephalins compete for the opiate receptors with affinities similar to morphine. Na^{+} and Mg^{2+} affect the binding of enkephalins. This study also shows a time dependent degradation of enkephalins. Waterfield et al. (119) showed a cross tolerance between morphine and enkephalin in morphine dependent guinea pigs.

Neurotransmitters

Dole (32), Way and Shen (120), and Weinstock (121) review narcotic effects on neurotransmitters prior to 1970. Way and Shen (120) conclude epinephrine and nonepinephrine changes after MS administration are more likely due to stress and these neurotransmitters do not play a role in tolerance and development. They also conclude that dopamine and 5-hydroxytryptamine (serotonin) may be involved, but there is no conclusive evidence. Weinstock (121) in reviewing cholinesterase and acetyl choline finds a definite involvement in analgesia, but does not show any relation with tolerance or dependence. Dole (32) in reviewing

neurotransmitters states

> . . . these results do not reveal any basic relation between narcotic action and catecholamine function. Such an association may exist, but in studies of the whole animal or whole brain any hormonal effects specific to analgesia or tolerance are obscured by the nonspecific neuronal discharges related to stress and to the non-narcotic excitory action of narcotic drugs (p. 833).

A large amount of work has been done on effects of narcotics on neurotransmitters. Lal et al. (122) have shown the behavioral changes induced by morphine resemble those of neuroleptics (dopamine receptor blocking agents) but is not brought about by the same mechanism since the dopamine receptor is still sensitive to the neuroleptics in the presence of morphine (122, 123). Clouet and Iwatsubo (124) suggest that opiates do not act directly on DA receptors but may produce DA•adenyl cyclase receptor sensitivity. Kuschinsky (125) was unable to find evidence of DA•adenyl cyclase receptor sensitivity in rats tolerant to morphine.

Perez-Cruet, Thoa, and Ng (126) found an increase in 5-HT with acute morphine and heroin administration. Buxbaum et al. (127) also find an increase in 5-HT synthesis and 5-HT turnover in rats, but conclude 5-HT does not play a part in morphine analgesia. Cheney et al. (128) also find that 5-HT turnover in mice does not play a role in tolerance and dependence.

Wei (129) found that intraventricular administration of norepinephrine produced analgesia and behavioral effects similar to morphine.

Henwood et al. (130) and Lee and Fennessy (131) found that morphine administration altered levels of brain histamine, but the time course of alteration did not correlate with morphine analgesia and

using other agents to alter histamine function had no effect on morphine analgesia.

Satoh et al. (132) found acute administration of morphine blocked excitation by acetylcholine (ACh) or L-glutamine, an effect which was naloxone antagonizable. After chronic morphine administration, morphine no longer blocked ACh or L-glutamine stimulation. Satoh et al. (133) show that cortical neurons are supersensitive to ACh or L-glutamine after morphine administration. Mullin and Phillis (134) found no difference in ACh turnover in dependent rats, but found increased turnover of ACh during withdrawal. Costa et al. (135, p. 6) in their study concluded "morphine action on brain cholinergic mechanism does not stem from a direct modification by this drug of the dynamic equilibrium of ACh in cholinergic neurons."

Statement of the Problem

These studies show that cells in culture can be used to evaluate toxicity of drugs, and that both neuronal and non-neuronal cells in culture have shown phenomena which resemble tolerance, addiction, and withdrawal to opiates. The studies involving tolerance, addiction, and withdrawal on opiates have generally been short term and consisted of qualitative observations of growth and toxicity. None of the cell strains which were made tolerant or dependent to the opiates still exist for quantitative determinations or biochemical evaluation as to cellular changes or mechanism(s) involved in tolerance and addiction at the cellular level. In our study we chose non-neuronal cells to study tolerance and addiction since both neuronal and non-neuronal lines have shown tolerance and addiction phenomena and because non-neuronal cells

are easier to grow and maintain than neuronal lines. We hoped to develop tolerant and addicted lines as a model and to qualitatively and quantitatively characterize these cells.

The methods we will use for characterization will include time lapse cinemicrophotography to qualitatively and quantitatively measure membrane movement, plating assays to quantitate toxicity, and uptake and incorporation studies to study the effects of opiates on uptake and incorporation of precursors for nucleic acid and protein.

Time Lapse Cinemicrophotography

The membrane serves as a primary regulator in cell function (diffusion barrier, specific transport, and binding). Morphine binding sites are also believed to occur on the plasma membrane (89, 90). Hoss and Smiley (136) show membrane lipids can immobilize opiates. Ouabain and theophilline effects on membranes of drug sensitive and drug resistant cell lines were studied by Lelievre et al. (137). Puck, Waldren, and Hsie (138) used time lapse cinematography to study the effects of 3'-5' cAMP and testosterone on CHO-K., S_3-HeLa, and V79-CHL cells. Nicolson, Smith, and Poste (139) showed that local anaesthetics affect membrane associated cytoskeletal organization in BALB/3T3 cells.

Pomerat (140) used time lapse cinematography to study membrane and cellular movements. Rose (141) also used time lapse cinematography to study membrane, cytoplasmic, and nuclear movements of KB and HeLa cells in different environments. Yang, Strasser, and Pomerat (142) studied vacuolization induced by various drugs (atropine, procaine, procaine amide, cocaine, pilocarpine, and ephedrine). Vacuolization did not affect cell motility or cell division and was reversed by

removal of the drug. Puromycin (143), colchicine (144, 145) and colchemid (146, 147) inhibition of cell motility have been studied using time lapse cinematography. Booker et al. (148) used time lapse cinematography to study differentiation in neuroblastoma clones.

These observations of the effects of drugs on membranes, cellular motion, and vacuolization both directly and using time lapse cinematography have been qualitative studies. Since morphine is believed to interact at the membrane, the utilization of time lapse cinemicrophotography on the membrane can be used to measure morphine effects on membrane movement in a qualitative and quantitative manner.

Plating Assay

Another method of quantitatively determining the toxicity of drugs to cells is by determining the effect the drug has on the ability of the cells to grow. Malcolm, Pringle, and Fisher (149) use a modification of Ham and Puck's (150) single cell plating technique to study the effects of Cd^{2+}, Zn^{2+}, Ca^{2+}, Se^{2+}, Cr^{4+}, rotenone, and nitrilotriacetate on the plating efficiency of T4 cells. They measure toxicity by a relative plating efficiency (RPE) which is defined as [(number of colonies in experimental)/(number of colonies in control)] x 100. This type of assay can be used to measure toxicity of drugs to any cells which form colonies when plated.

Uptake/Glass Fiber Filters

Experiments measuring transport and/or incorporation of radioactive precursors into cultured cells require replicate cultures or samples for the analysis of the time course, effect of various inhibitor con-

centration, or other parameters. Cells grown in suspension can easily be labeled, collected by filtration, and selectively treated (151). Replicate monolayer cultures can also be grown on coverslips (152), plastic discs (153), and on the bottom surface of glass scintillation vials (154, 155).

The synthesis of specific macromolecules can be measured by determining the incorporation of specific radioactive precursors into acid insoluble material (156). [^{3}H]-Thymidine is incorporated into DNA, [^{3}H]-uridine into RNA, and [^{3}H]-tyrosine into protein. The difference between uptake and incorporation is the amount transported into the cellular pool (157).

Various perturbations can be studied by the effects that they have on incorporation of these precursors. Painter (158) used stimulation of thymidine uptake as a measure of drug induced DNA damage. Hydroxyurea has been shown to inhibit the uptake and incorporation of [^{3}H]-thymidine into DNA (159-161). Dybing (39) found alanine, AIB, hypoxyxanthine, and thymidine uptake was inhibited by chlorpromazine. Howard et al. (162) found thymidine uptake was stimulated by the addition of conditioned media. Ooka and Daillie (163) found incorporation of [^{3}H]-thymidine into KB and HeLa cells was temperature dependent.

To determine the uptake and incorporation the cells or labeled precipitate must be separated and collected. Many authors (164-168) have used cellulose filters, glass fiber filters, or membrane filters to collect the cells or precipitates. Ball, Van den Berg, and Poynter (169) are critical of using filters to collect precipitate due to lack of quantitative transfer.

CHAPTER II

MATERIALS AND METHODS

Materials

HeLa cells (a human epidermal carcinoma (cervical) established by Gey *et al.* (170) in 1952) were purchased from American Type Culture Collection Cell Repository (ATCCR) as CCL-17 KB cells (CCL-17 KB has been identified by ATCCR as a HeLa contaminant). LM cells, CCL 1.2 (fetal mouse lung fibroblast) were also purchased from ATCCR. Calf serum (10 days to 6 months) was purchased from Microbiological Associates. Culture media (199 and McCoy's 5A) were purchased as powders from Grand Island Biological Co. Potassium penicillin G was purchased from Squibb and dihydrostreptomycin purchased from Pfizer. $NaHCO_3$ was purchased from Fisher Scientific. Stock medium 199 was prepared at a 5X concentration and stock McCoy's 5A was prepared at a 2.5X concentration; stock media were sterilized by vacuum filtration, divided into aliquots, and stored frozen. Stock penicillin and streptomycin solutions are prepared at 10000 U/ml and 5000 µg/ml respectively, sterilized by filtration and stored frozen. Working solutions of medium 199 are prepared by mixing 100 ml of frozen stock 199, 50 ml of calf serum, 5 ml of penicillin and streptomycin (final concentration of 100 U/ml and 50 µg/ml respectively), and 1.8 ml of sterile $NaHCO_3$ (0.11 mg/ml). Volume is brought up to 500 ml with sterile glass distilled water.

Working solutions of McCoys 5A are made by mixing 200 ml of stock McCoy's 5A, 50 or 30 ml of calf serum (10% or 6% serum), 5 ml of penicillin and streptomycin stock solution, and 8.0 ml of sterile $NaHCO_3$ and made up to 500 ml using sterile glass distilled water.

Drugs were obtained from the following sources: Eli Lilly and Co. - acetylsalicylic acid, codeine sulfate, propoxyphene, and morphine sulfate; Sigma - amphetamine sulfate; Parke-Davis and Co. - diphenhydramine-HCl; Calbiochem. - caffeine; U. S. Industrial - ethanol; National Institute of Health - heroin; American Quinine Co. - meprobamate; Merck, Sharp, and Dohne - nalorphine; Matheson, Coleman, Bell - phenacetin; Eastman Organic Chemicals - isoamyl alcohol, phenethyl alcohol; Wyeth - phenobarbital; Roche - diazepam, levorphanol, and dextrorphan, and Abbot - procaine. All drugs were made up as needed in sterile glass distilled water or glass distilled water with 1% dimethylsulfoxide (a concentration that was independently determined to be non-toxic) and then sterilized by membrane filtration.

Radioisotopes used in these studies were obtained from the following sources: Schwartz-Mann - [methyl ^3H]-thymidine, specific activity 1.9 Ci/m mole and [5-^3H]-uridine, specific activity 2.0 Ci/m mole; Amersham - D,L-[U-^3H]-tyrosine, specific activity 0.43 Ci/m mole and [1(n)-^3H]-morphine, specific activity 28 Ci/m mole. The [^3H]-morphine was further purified by thin layer chromatography on silica gel G-250 using methanol:benzene:water (75:10:15, v/v/v) and compared to morphine standard (171). The [^3H]-morphine peak was scraped off the thin layer chromatogram and extracted 4X with 0.5 ml of ethanol (95%).

Other materials used in these studies were obtained from the following sources: Matheson, Coleman, and Bell - 2-amino-2-(hydroxy-

ethyl)-1,3-propanediol (Tris); Fisher Scientific Co. - NaCl, KCl, Na_2HPO_4, KH_2PO_4, phenol red, methanol, ethyleneglycol, and p-dioxane; J. T. Baker Chemical Co. - $CaCl_2$, $MgSO_4 \cdot 5H_2O$; Sigma Chemical Co. - dextrose, sucrose; Eastman Organic Chemical - napthalene; Packard Instrument Co. - 2,5-diphenyloxazole (PPO) and 1,4-bis-2(5-phenyl-oxazolyl)-benzene (POPOP). Petri dishes for cell culture use (plastic, 60 x 15 mm with 2 mm grid #3030) were obtained from Falcon Plastics. Coverslips were obtained from Corning (Corning #1, 2.4 cm). Filters were obtained from the following sources: Whatman - GF/A, #1321-014, 24 mm and GF/B, #1321-077, 24 mm; Reeves Angel - glass fiber filters, #934 AH, 24 mm; and Millipore - membrane filter, HAWG 0 24-00, 0.45 μ, 25 mm.

Methods

Stock Cell Lines

HeLa cells were grown as monolayer cultures at 37° in either milk dilution bottles (MDB) or Falcon plastic tissue culture dishes using medium 199 supplemented with 10% calf serum, penicillin, and streptomycin. The LM cells were cultured as monolayers in MDB using McCoy's 5A medium supplemented with 10% calf serum, penicillin, and streptomycin or as suspensions in 125 ml Erlenmyer flasks containing 20-30 ml of McCoy's 5A supplemented with 6% calf serum, penicillin, and streptomycin. Suspension cultures were maintained using a New-Brunswick G-10 gyrotory shaker at 50 oscillations per minute in a 37° room.

Plating Assay

Stock HeLa cells grown in a MDB are scraped, suspended (using a

pipette), and centrifuged in a clinical centrifuge at 3000-5000 RPM. Cell pellet is suspended in phosphate buffered saline (172) pH 7.2 for counting on a Coulter counter (Coulter Electronics, model B, 100 μm orifice) or in medium 199 for haemocytomer counting. Cells are centrifuged as before. The cell pellet is suspended in medium 199 and diluted with medium 199 to give 1 x 10^3 cells/ml. One ml of the cell suspension (1 x 10^3 cells) is then plated into a petri dish containing 4.0 ml of 199 + 12.5% calf (control) or 4.0 ml of 100 + 12.5% calf + drug (1.25 x final concentration) to give a final volume of 5.0 ml of 199 with final serum concentration 10%. Drug solutions are made up as in materials and mixed on a 1:1 (v/v) basis with 2X (medium 199 + 12.5% calf) to keep medium concentration uniform. Plates are incubated for one week at 37° in a humidified CO_2/air (5%/95%) incubator (National, model 3321). The medium is removed by aspiration, plates washed with Hanks' BSS (173), stained with 0.5% aqueous crystal violet, rinsed, and air dried. Colonies consisting of three or more cells are counted using a microscope (Baush and Lomb, RD9742) at 45X magnification. Cultures incubated without drugs are used to establish the control plating efficiency (taken as 100%). Absolute plating efficiency under these conditions is 60%. The number of colonies on the plates with drug is compared to the control using a relative plating efficiency (RP = (number of colonies in experimental)/(number of colonies in control) x 100). Using a plot of log RP Vs drug concentration, the dose of each drug that inhibits plating by 50% (RP_{50}) is obtained.

Development of Cells with Increased Tolerance

The mechanism(s) by which cells in culture with increased tolerance

arise are unknown. The procedure used in this study to obtain these cells is described below.

The first approach used was the inoculation of 1.5×10^3 cells into a MDB containing RP_{50} of the respective drug. After 3-4 passages (7-10 days each), there were insufficient multiplying cells to allow continuation of the cultures.

The next approach used the protocol shown in Table III. In this protocol cells were started at 1.5×10^3 cells/MDB using 0.1 RP_{50} of the drug. Cells were grown to confluency (7-10 days) and subcultured the indicated number of times. The cell number was then reduced to 1.5×10^3 cells/MDB and the cells exposed to the next concentration of the drug and the procedure repeated. After reaching 1.0 RP_{50}, cells were maintained by subculturing using a 1:2 or 1:3 split in 1.0 RP_{50} of the drug (0.5 RP_{50} was reserve to use if the cells at 1.0 RP_{50} were unsatisfactory).

Measurement of Cell Attachment

To determine if the drugs affected the attachment of HeLa cells, a modification of the plating assay was used. In this modification, HeLa cells (10^3) were plated as described above with plates containing 1.5 RP_{50} of the drug. At various times the medium (containing any unattached cells) is removed and the plate washed 2X with 5.0 ml of Hanks BSS (173). Five ml of 199 + 10% calf is then added and the plates incubated, washed, stained, and counted as above. The number of colonies attached in 24 hours on a control plate (in the absence of drug) is taken as the maximum and other time points are represented as

TABLE III

PROTOCOL FOR DEVELOPMENT OF TOLERANT CELLS

Step	Drug Concentration	Initial Inoculation (cells/MDB)	Times Subcultured
1	0.1 RP_{50}	1.5×10^3	2
2	0.5 RP_{50}	1.5×10^3	2
3	1.0 RP_{50}	1.5×10^3	10
4	1.0 RP_{50} - maintenance		

the percent of maximum attached. Drug toxicity is checked by a plate with 1.5 RP_{50} of the drug grown for a week as above.

Growth of Cells in Suspension

Stock LM cells are harvested, suspended, centrifuged, and counted using a Coulter counter. Cells are then suspended at 0.5 x 10^5 cells per ml in McCoy's + 6% calf or McCoy's + 6% calf + drug (20 ml total volume). At various time points following inoculation (t = 0) aliquots (0.2 ml) are removed, suspended in 19.8 ml of counting fluid (0.9% saline) and counted on a Coulter Counter to determine cell concentration. Duplicate flasks are run for each drug concentration with triplicate determinations at each time point.

Cinematography

HeLa cells in medium 199 + 10% calf were inoculated into a Dvorak-Stotler perfusion chamber and incubated inverted in a 5% CO_2/95% air incubator at 37° for 4 h to allow attachment. The chamber was then placed on the stage of a Leitz laboratory model SM-M phase microscope and the temperature maintained at 37° using a Sage Model 279 air curtain incubator. Cells were perfused with 199 + 10% calf or 199 + 10% calf + drug at 0.99 ml/h using a Sage Model 341 syringe pump. Time lapse cinematography was done using a Sage series 500 cinemicrography system using Kodak 16 mm Tri-X reversal film (#7278) at various rates.

Binding of [^{3}H]-Morphine

Binding of [^{3}H]-morphine was measured using the method of Klee and

Nirenberg (76). Cells were prepared as before and suspended in 0.01 M Tris, 0.32 M sucrose at pH 8.0 (on ice). The cell suspension was then incubated at 37° with [^{3}H]-morphine in the presence of morphine, dextrorphan, or levorphanol. Cells were collected on a Whatman GF/B glass fiber filter under vacuum aspiration (25 mm Hg) and rinsed 2X with 7.0 ml of Hanks BSS (173). The cells were then air dried and binding measured by liquid scintillation counting on a Packard Tri Carb, model 3320, using Bray's cocktail (174).

Displacement of bound morphine was measured by incubating cells in Tris-sucrose buffer at 37° for 10 min in 10^{-8} M morphine + [^{3}H]-morphine. Cells are pelleted by centrifugation and media removed by aspiration. Cells are then suspended in Tris-sucrose buffer containing 10^{-8} M drug at 37° for desired time, collected, washed, and counted as above.

Growth on Glass Fiber Filters

Support systems (glass fiber filters, milk dilution bottles, membrane filters, and coverslips) were sterilized by autoclaving in glass petri dishes. Scintillation vials and caps were wrapped in foil (caps wrapped separately) and autoclaved.

HeLa cells were scraped, counted, and suspended in 199 + 10% calf (4×10^3 to 4×10^6 cells/ml). Cells (usually 1×10^5) are plated on support (generally GF/A filter) in 0.25 ml total volume (1.0 ml for scintillation vials, 2.0 ml for MDB) and incubated 4 h at 37° in a humidified CO_2/air (5%/95%) water jacketed incubator (National, model 3321). Medium 199 + 10% calf serum was added to a final volume of 10 ml (5.0 ml for scintillation vials) and further incubated for the

indicated time (usually 20 h, but this is not required). The appropriate radioactive precursor is added ([^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-tyrosine) and mixed by gentle swirling. After the requisite time of incubation (depends on experiment) the filter is removed to a Sargent S-35620 polypropylene Buchner funnel on a suction flask and washed twice with 15.0 ml of Hanks BSS (173) (uptake measurement) or twice with 15.0 ml of 10% trichloroacetic acid (incorporation measurement). Aspirator vacuum filtration (25 mm Hg) was used. The filters were removed to glass scintillation vials, air dried, and counted on a Packard Tri Carb using Bray's scintillation cocktail as before.

In experiments to determine proportionality to cell number, 1 x 10^3 - 1 x 10^6 cells are plated/support, incubated 24 h, and pulsed with [^{3}H]-precursor (0.5 µCi/ml), terminated, and counted as above. For proportionality to precursor concentration, 1 x 10^5 cells are plated/filter, incubated (24 h), and pulsed with [^{3}H]-precursor (0.05 - 5 µCi/ml) for 4.0 h, terminated and counted as above. For experiments designed to measure duration of pulse, 1 x 10^5 cells are plated/filter, incubated (24 h), and pulsed at 0.5 µCi precursor/ml for varying lengths of time, terminated, and counted as above.

Hydroxyurea Inhibition

Whatman GF/A filters are prepared as above. HeLa cells prepared as above are plated at 1 x 10^5 cells/filter (0.25 ml), attached and grown as above. After growth period, 2.0 ml of media is removed and replaced with either 2.0 ml of 199 + 10% calf or 2.0 ml of 100 + 10% calf containing hydroxyurea at the appropriate concentration. Cultures were then pulsed with 5 µCi of [^{3}H]-thymidine (0.5 µCi/ml) for 4.0 h.

Pulse is terminated and cells counted as above.

Plasma Membrane Isolation

HeLa cells are scraped, suspended, and washed 4X with Hanks BSS. Membranes are prepared using Shin and Carraway's (175) and Carraway et al.'s (176) modifications of a Zn^{2+} stabilization method (177). In this method cells are swollen (2X, 10 min each) in 50 mM Tris, pH 7.4 (ice cold) and centrifuged at 3000 RPM to pellet swollen cells. Cells are then centrifuged, suspended in 5.0 ml of 40 mM Tris, pH 7.4 (on ice) and homogenized using a Dounce homogenizer with tight pestile. Cells are monitored visually to assure disruption. A small volume of homogenate is saved for enzyme assays, and the remainder washed 2X in ice cold 40 mM Tris, pH 7.4 (centrifuged 10 min at 5000 RPM). Cells are suspended in ice cold 40 mM Tris, pH 7.4 and centrifuged at 750 RPM to remove nuclei (2X). The supernatant solution is centrifuged 10 min at 5000 RPM to pellet the plasma membrane and other membranes. The pellet is suspended in 2-5 ml of 40 mM Tris, pH 7.4 and layered on top of a discontinuous sucrose gradient (55-50-45-40% sucrose, w/v) and centrifuged at 15000 RPM in a L-5-65 Beckman centrifuge using a SW-27 head for 60 min at 4°. The plasma membrane (interfaces 2,3 (40/45 and 45/50 interfaces)) is collected, washed 2X with 40 mM Tris, pH 7.4, centrifuged, and suspended in 50 mM Tris, 0.32 M sucrose (pH 7.0) and stored frozen until analysis.

Membrane protein was determined by the method of Lowry et al. (178). Membrane purity was checked by the use of enzyme marker assays. Glucose-6-phosphatase was used as an endoplasmic reticulum marker (179). Succinic INT reductase as a mitochondrial marker (180), and 5' nucleo-

tidase as a plasma marker (180). The inorganic phosphate was measured using a modification of the method of Lazarus (181) using 1 ml of isoamyl alcohol to extract the phosphomolybdate. The absorbance of the alcohol phase was measured at 740 nm.

SDS Acrylamide Gel Electrophoresis

Membrane samples were dissolved by boiling for 5 min in 4% SDS-1% mercaptoethanol-5 mM EDTA-50 mM phosphate (pH 7.4) at a protein concentration of 3-10 mg/ml, and then incubated overnight at 37°. One tenth volume of glycerol-0.01 mg bromophenol blue was added and a sample 50-300 µg of protein/gel applied to one cm 5% gels. Gels are run 45 min to remove persulfate, samples loaded, and gels run at 8 m AMP/gel until tracking dye reached the end of the gel (~ 5 h). Gels are stained for protein and carbohydrate by the method of Fairbanks et al. (182).

CHAPTER III

ASSESSMENT OF TOXICITIES OF DRUGS

Plating Assay - HeLa Cells

Before one can utilize cultured cells for developing cell lines which show phenomena resembling tolerance and addiction, the toxicity of the drug to the cell lines used must be measured. For HeLa cells, which will attach, grow, and form individual colonies when plated as single cells into plastic petri dishes, the toxicity of a drug can be measured by the effect that it has on colony formation. Toxicity can be measured as a decrease in the number of colonies formed when compared to the control or as morphological changes, specifically as changes in the size or shape of cells and colonies. Figure 2 shows the dose response of HeLa cells to codeine, morphine, and heroin. Stock HeLa cells were plated according to the procedure given in materials and methods in the presence of the indicated drug concentrations. Relative plating efficiency (RP) is plotted vs. drug concentration used. Absolute plating efficiency under these conditions is 60%. With codeine, there was inhibition of plating at all drug concentrations used, while there was a threshold concentration with morphine and heroin below which little inhibition (< 10%) was observed. From similar curves obtained from all the drugs, three concentrations are recorded in Table IV: first, the highest concentration which has

Figure 2. Dose Response of Stock HeLa Cells to Codeine, Morphine, and Heroin

Stock HeLa cells were plated according to the procedure given in the Materials and Methods section in the presence of the indicated drug concentrations. The relative cell plating efficiency (number of colonies formed in presence of drug x 100/number of colonies formed in absence of drug) is plotted on semilogarithmic graph paper against mM drug concentration. Part A, codeine; part B, morphine; and part C, heroin. Platings were done in triplicate. The error bars show the standard deviation.

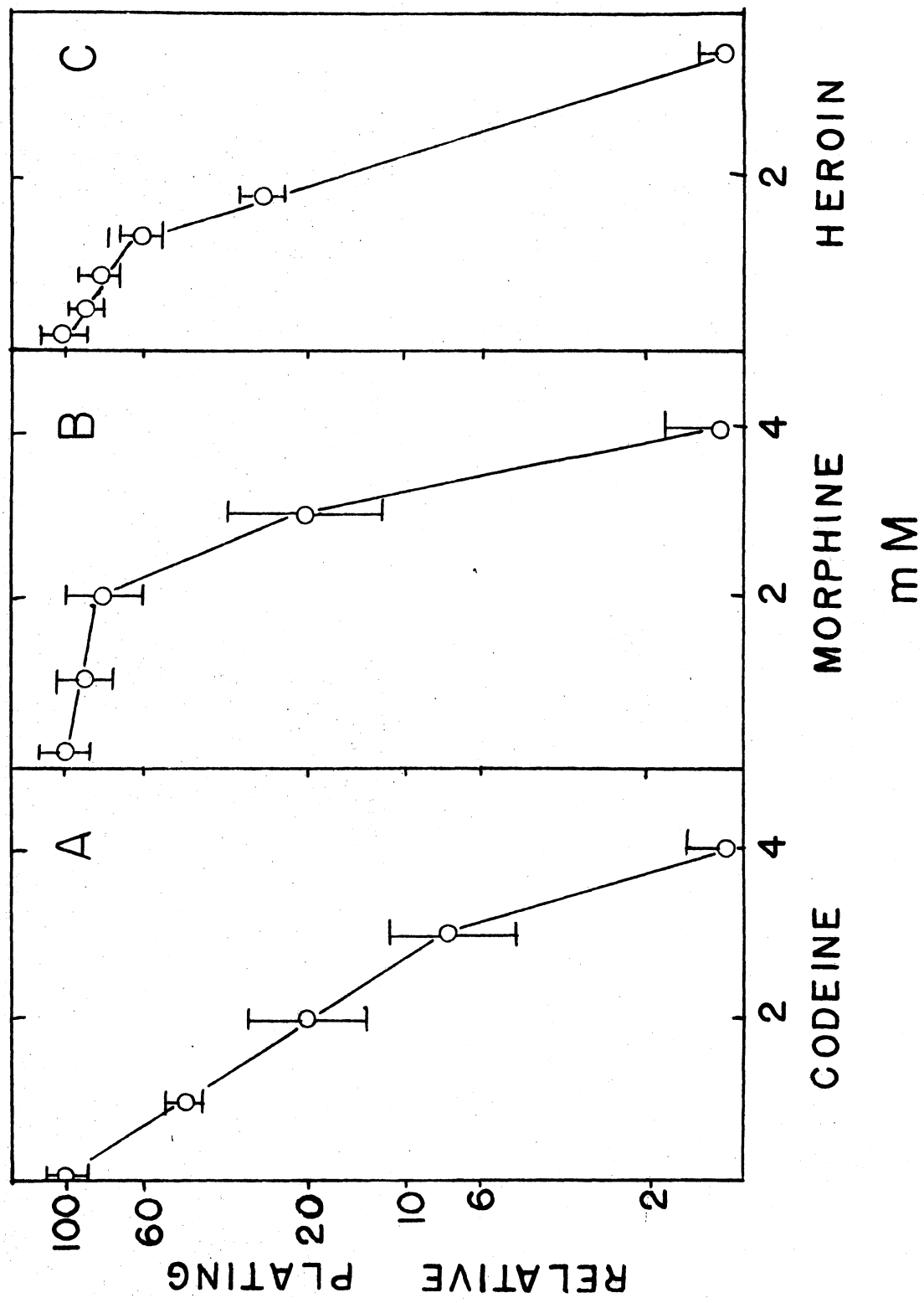

RELATIVE PLATING
100
60
20
10
6
2
A
B
C
2
4
2
4
2
CODEINE
MORPHINE
HEROIN
mM

TABLE IV

EFFECT OF DRUGS ON HELA PLATING

Drug	mM Drug Concentration for		
	< 10% Inhibition	50% Inhibition	> 90% Inhibition
Acetylsalicylic Acid	0.5	1.1	2.5
Amphetamine	2.0	3.5	5.0
Caffeine	0.5	2.5	5.0
Codeine	-	1	4
Dextrorphan	0.08	0.51	1.0
Diazepam (Valium)	0.025	0.065	0.1
Diphenhydramine HCl (Benadryl)	0.2	0.24	0.30
Ethanol	1.5	2.7	NR*
Heroin	0.65	1.3	1.95
Levorphanol	0.05	0.37	0.9
Leucine Enkephalin	0.03	0.13	0.22
Meprobamate	1	2.5	NR
Morphine	2	2.5	4
Nalline	0.005	0.0275	0.06
Phenacetin	1.4	2.1	NR
Phenethylalcohol	0.2	1.2	2.5
Phenobarbitol	1	1.3	2.2
Propoxyphene (Darvon)	1	2.5	NR

*NR means 90% inhibition not reached with concentrations used.

little or no effect on the relative plating (< 10% inhibition); second, the concentration which decreases the relative plating 50%, that is, an RP_{50}; finally, the concentration which produces 90% or greater inhibition. With several drugs (propoxyphene, ethanol, meprobromate, and phenacetin) 90% inhibition was not reached with drug concentrations tested.

Growth Assays - LM Cells

LM cells do not form individual colonies when plated as single cells, so to measure the toxicity one must look at total cell number. LM cells adapt well to suspension cultures which allows for easy measurement of the effects of the drugs on the growth rate of the cells. In this assay, LM cells were inoculated as described in Materials and Methods. At various times after inoculation, the culture was sampled and cell concentration determined with the Coulter Counter (triplicate determinations on duplicate samples). The dose responses of LM cells to morphine (Part A) and heroin (Part B) are shown in Figure 3. Since these cultures are maintained and sampled over time, a dynamic representation of the effect of each concentration of drug is obtained (for the plating assay, the colonies formed are measured at one time point, i.e., 7 days, and dynamic effects are lost). Part C of Figure 3 shows the cell number vs. heroin concentration at 80 h in a fashion similar to the static plot for HeLa plating. Using this type of plot, the concentrations of drugs which has little or no effect on cell number (< 10%), the concentration which reduces cell number 50%, and the concentration which reduces cell number by 90% are obtained for various drugs. These concentrations are listed in Table V. The LM

Figure 3. Dose Response of LM Cells to Morphine and Heroin

Part A. The concentrations of morphine used were: 0, 0; ●, 0.625 mM; ⊖, 125 mM; and ⊖, 2.5 mM.
Part B. The concentrations of heroin used were: 0, 0; ●, 0.5 mM; ⊖, 1 mM; and ⊖, 2 mM.
Part C. A plot of cell number at 80 hours against heroin concentration.

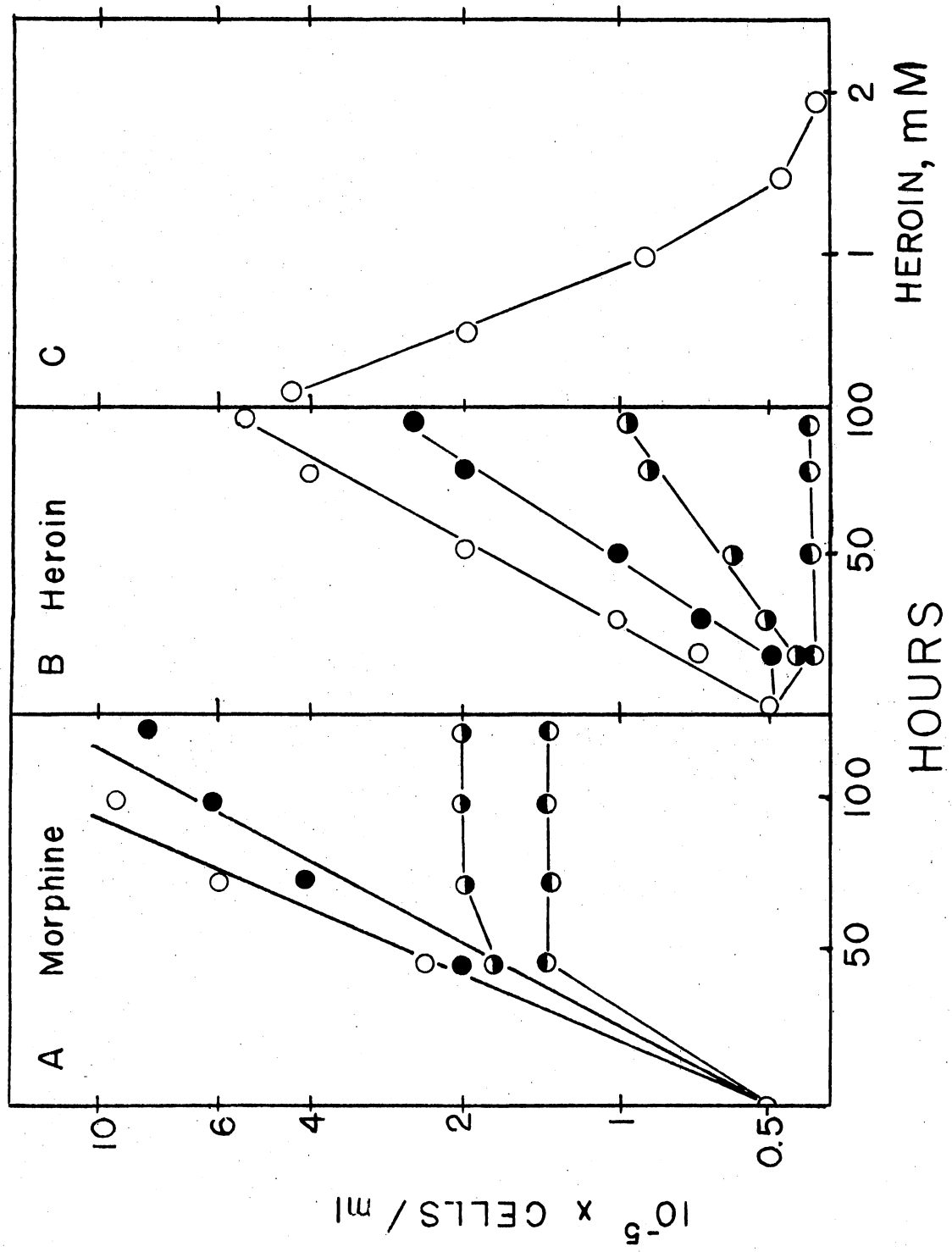

A Morphine
B Heroin
C
10
6
4
2
1
0.5
50
100
50
100
1
2
HOURS
HEROIN, mM
10^{-5} x CELLS / ml

TABLE V

EFFECTS OF DRUGS ON LM SUSPENSION CULTURES

Drug	mM Drug Concentration for		
	< 10% Inhibition	50% Inhibition	> 90% Inhibition
Amphetamine	0.6	1	1.25
Caffeine	0.625	2	2.5
Codeine	0.2	1	NR*
Diphenhydramine HCl	0.06	0.125	0.25
Ethanol	1	2	8
Heroin	0.1	0.5	1.6
Morphine	0.5	1	1.25

*NR means 90% inhibition not reached with highest concentrations used.

cultures were more sensitive to the drugs in suspension than in the HeLa plating assay. This can be due to one of two effects; increased sensitivity of cells in suspension to the drugs as opposed to cells growing on a solid surface; or due to an inherent increased sensitivity of LM cells over HeLa cells.

Development and Assessment of Tolerant Cell Lines

Growth Procedure

The first procedure used was to inoculate 1.5×10^3 cells into a MDB containing RP_{50} of the respective drugs (the concentrations listed for 50% inhibition in Table IV). After 3-4 passages (7-10 d each), there were insufficient multiplying cells to allow continuation of the cultures.

The next protocol used was that given in Table III in the methods section. This involved inoculating 1.5×10^3 HeLa cells/MDB containing 0.1 RP_{50} of the drug, growing cells to confluency, and subculturing (2X, 1:2 split) in medium containing 0.1 RP_{50} of the drug. These cells were then inoculated at 1.5×10^3 cells/MDB containing 0.5 RP_{50}, grown to confluency, and subcultured (2X) in medium containing 0.5 RP_{50} of the drug. These cells were then inoculated at 1.5×10^3 cells/MDB containing 1.0 RP_{50} of the drug, grown to confluency, and subcultured (10X) in medium containing 1.0 RP_{50} of the drug. After this point, cells were put on a maintenance schedule which involves subculturing (1:2 or 1:3 split) at 1.0 RP_{50} and 0.5 RP_{50} of the drug. The 0.5 RP_{50} cultures served as backup cultures if cultures grown at 1.0 RP_{50} were unsatisfactory.

Assessment of Tolerance

Cultures grown in the above manner were periodically tested for tolerance using the plating assay described in the materials and methods section. Tolerance was determined after 130, 240, 320, 415, and 645 days of culture. The relative plating efficiency was obtained by comparing the number of colonies produced in the presence of a particular drug to the number of colonies formed by the same number of cells plated in the absence of any drug. Figure 4 shows the variation in tolerance to morphine with length of cultivation in the presence of drug. At 130 days there is a slight but not significant increase in tolerance is seen at 240 days. A further increase is observed at 415 days, but the level of tolerance after 645 days is unchanged from that at 415 days, so it appears that a maximal tolerance is achieved.

Figure 5 shows the response of HeLa cells to varying concentrations of diphenhydramine hydrochloride (benadryl). Cells grown in the presence of diphenhydramine hydrochloride for 645 days show less sensitivity to the drug than do control cells.

Table VI shows the development of tolerance at various times during drug exposure. Cultures grown in the presence of the corresponding drug are more tolerant to amphetamine, diphenhydramine hydrochloride (benadryl), codeine, meprobromate, and morphine as is seen by increased plating efficiencies. Cultures grown in the presence of caffeine show no increase at 1.0 RP_{50} concentrations, only a slight increase in tolerance at 1.5 RP_{50} concentrations whereas cultures grown in the presence of propoxyphene (Darvon) show no increase in tolerance. With the meprobromate and amphetamine cultures, a decrease

Figure 4. Variation in Tolerance to Morphine with Length of Cultivation in the Presence of Drug

Days of growth in presence of morphine 0, ○; 130, ●; 240, ◐; 415, ◑; and 645, ◒.

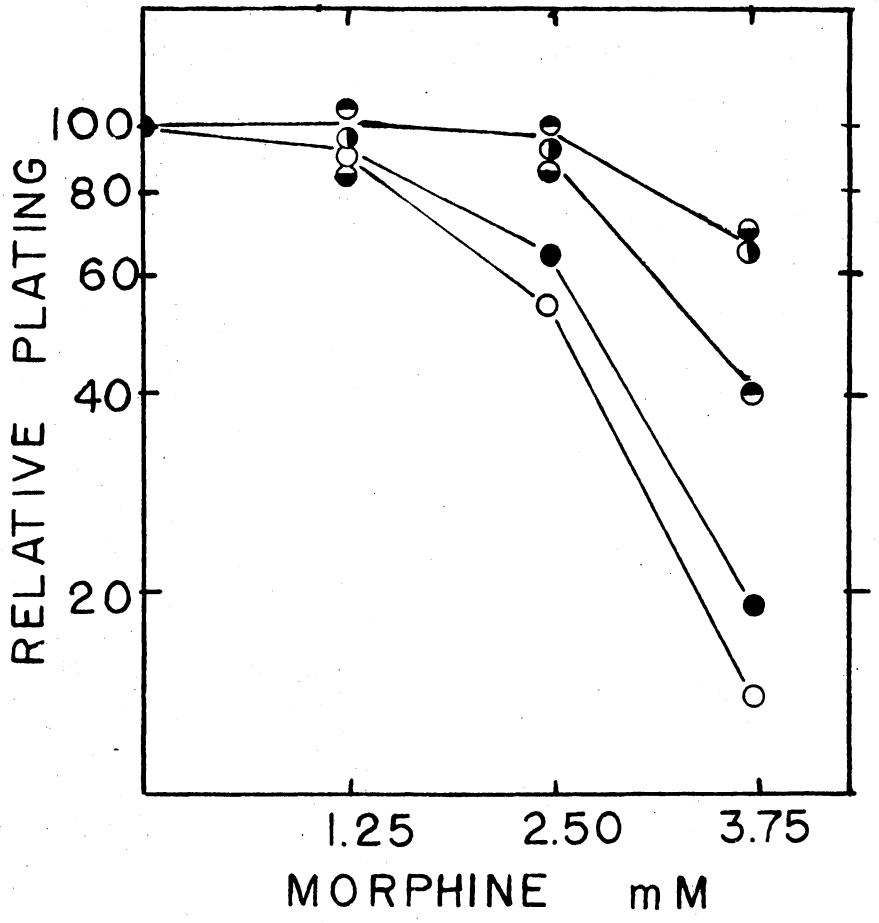

RELATIVE PLATING
100
80
60
40
20
1.25
2.50
3.75
MORPHINE mM

Figure 5. Effect of Prior Growth in Drug-Containing Medium on Growth in the Presence of Varying Concentrations of Diphenhydramine Hydrochloride

Control HeLa cells (O) and cells grown in the presence of RP_{50} of diphenhydramine hydrochloride for 645 days (●) were exposed to the indicated concentrations of diphenhydramine hydrochloride as multiples of the RP_{50} concentration. The relative plating (from triplicate plates) is plotted on the ordinate. The error bar shows the standard deviation.

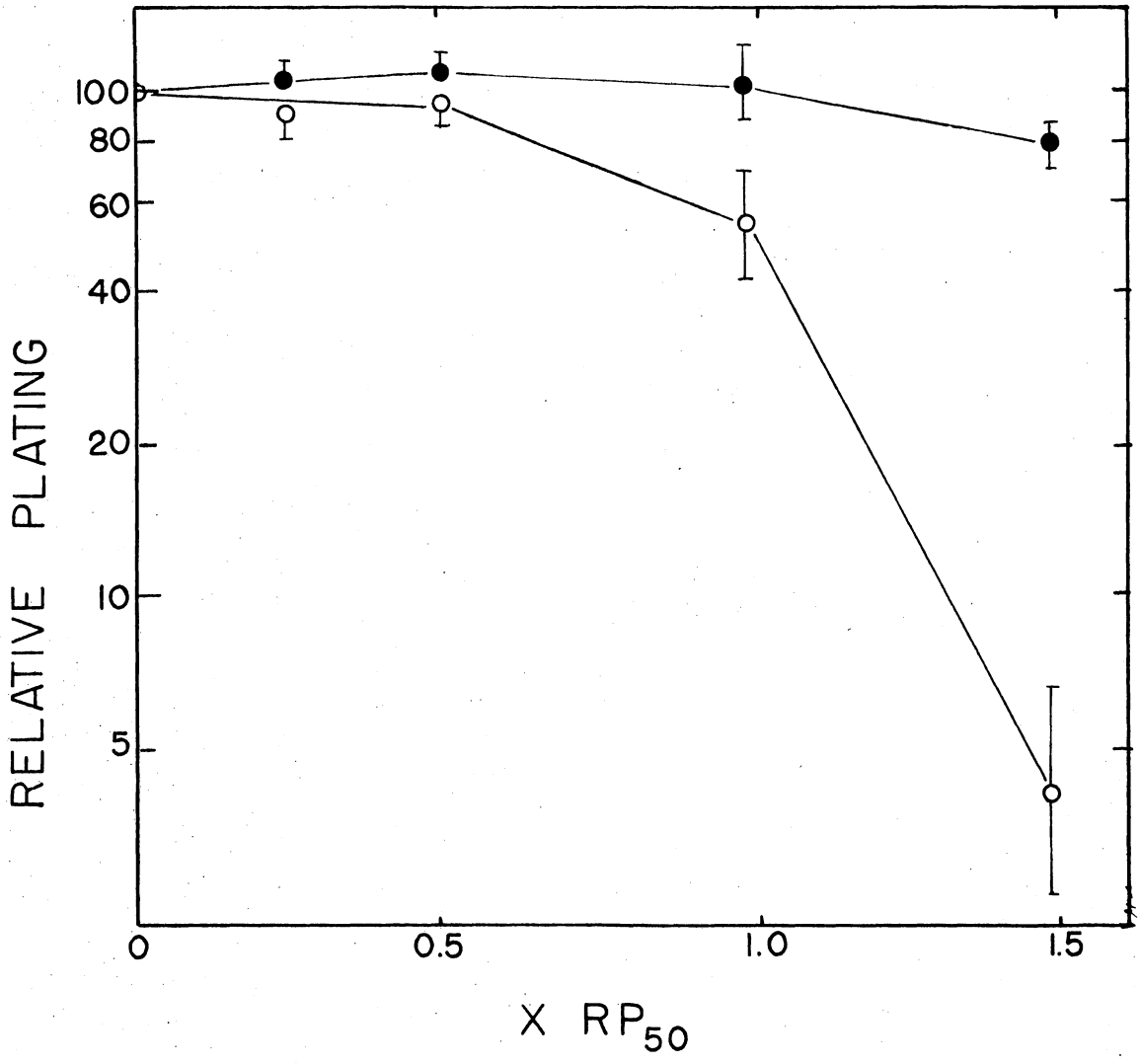

RELATIVE PLATING
100
80
60
40
20
10
5
0
0.5
1.0
1.5
X RP50

TABLE VI

DEVELOPMENT OF TOLERANCE AT VARIOUS TIMES
DURING DRUG EXPOSURE

Drug	Drug Concentration RP_{50}	Relative Plating* Length of Exposure, Days 0	130	240	415	645
Amphetamine	1.0	31	78	102	105	78
	1.5	12	27	43	102	45
Caffeine	1.0	55	51	64	72	51
	1.5	31	30	48	57	51
Codeine	1.0	88	83	55	99	92
	2.0	44	79	15	79	85
	3.0	4	59	2	52	74
Diphenhydramine HCl (Benadryl)	1.0	41	79	104	118	108
	1.5	7	41	63	107	79
Meprobamate	0.5	101	-	102	102	-
	1.0	73	89	102	98	-
	1.5	10	64	57	26	-
Morphine	0.5	91	-	109	85	96
	1.0	55	65	100	89	95
	1.5	14	19	40	70	67
Propoxyphene	1.0	68	-	45	70	-
	1.5	49	-	0	50	-

*Relative Plating = $\frac{\text{number of colonies in presence of drug}}{\text{number of colonies in absence of drug}} \times 100$

Relative plating at various times during training. HeLa cells trained for indicated time (control HeLa = 0) are plated in presence of indicated drug concentration.

of tolerance is seen at 645 days when compared to the levels seen at 415 days. The meprobromate culture was lost at the 645 day testing.

Cross Tolerance to Heroin

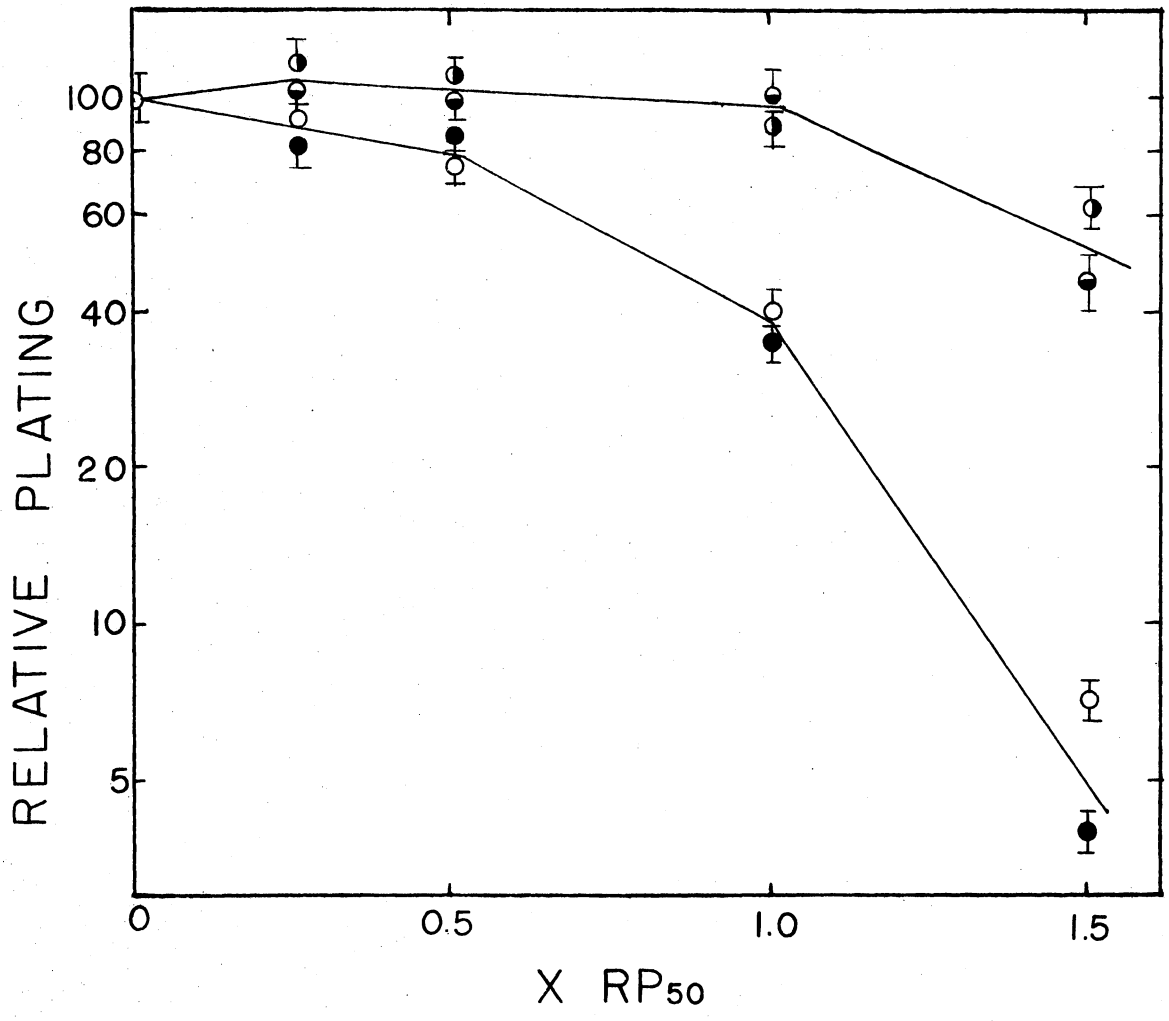
Because of the biotransformation of heroin into morphine (by de-acylation, heroin → monoacetylmorphine → morphine), their chemical structure relations, and competition for the same receptor sites, cell lines tolerant to morphine would also be expected to show tolerance to heroin. This cross tolerance is seen in Figure 6. Control HeLa cells show the same relative sensitivities to morphine as they do to heroin. In terms of absolute sensitivities, the HeLa cells are more sensitive to heroin than they are to morphine, an effect which is also seen in intact animals. This is probably due to the higher lipid solubility of heroin compared to morphine, which allows the heroin into the membrane at a higher rate. When cells which have been grown in the presence of morphine for 415 days are tested against various concentrations of morphine or heroin, equivalent tolerances are observed, indicating that morphine tolerant cells are also cross tolerant to heroin.

Membrane Motion

Qualitative observations showed that morphine reduced the movement of the cell's membrane when it was added to the medium. This was augmented by quantitating the membrane movement by the following procedure. Cells were inoculated, grown, and filmed in a Dvorak-Stotler perfusion chamber as described in the methods section. Either medium 199 + 10% calf serum or medium 199 + 10% calf serum + drug is perfused through the

Figure 6. Effect of Heroin on HeLa Cells Grown in Presence of Morphine for 415 Days

Control HeLa cells plated with heroin O and with morphine ●. Morphine-tolerant HeLa cells were plated in the presence of ⊖ morphine and ◐ heroin.


RELATIVE PLATING
100
80
60
40
20
10
5
0
0.5
1.0
1.5
X RP50

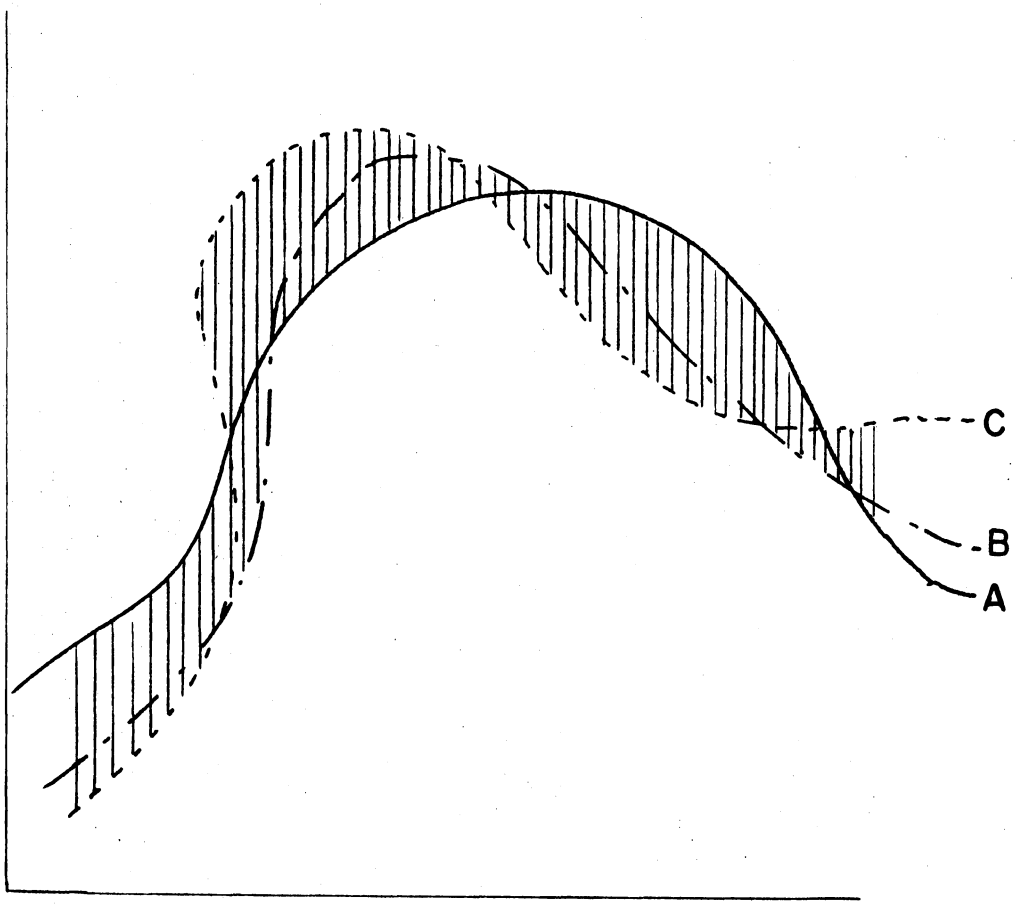
chamber during filming. For analysis, the film is projected onto tracing paper one frame at a time using a Lafayette Model AHP-300 16 mm analyzing projector. The outline of a cell area picked at random for being in sharp focus was drawn on the tracing paper and a flattened portion of the cell membrane chosen for analysis. The membrane position on three consecutive frames was traced for each of three cells. The filming rate used for this analysis was 15 frames/min; thus the measurements correspond to an 8 second time interval.

After the three outlines have been traced onto the paper, the total area between the outer lines on the drawings was measured using a planimeter. This is shown diagrammatically in Figure 7. Line A corresponds to the membrane position in frame 1, line B corresponds to the membrane position in frame 2, and line C corresponds to the membrane position in frame 3. The shaded portion represents the area through which the membrane has moved. This is done along a designated segment of membrane (about 100 mm contour length as projected which is equivalent to 40 μm of cell membrane). Both the area reading from the planimeter and the length of the segment measured were recorded and converted into square microns per micron. The system was calibrated using a ruled slide, known projection distances, and magnification.

The area through which the membrane moved on control cells was approximately 0.1 $\mu m^2/\mu m$ of membrane length. With morphine trained cells (415 days) the corresponding value in the absence of morphine was 0.08 $\mu m^2/\mu m$. This represents a statistically significant reduction at the 5% confidence level (student's t). Each experiment was repeated at least twice and three or four individual cells analyzed

Figure 7. Membrane Position as a Function of Time

Line A represents membrane position at frame 1, line B represents membrane position at frame 2, line C represents membrane position at frame 3. The shaded portion represents the area through which the membrane has moved during an 8 second interval.


C
B
A

at each concentration. The variation of movement among triplicate cells in a typical experiment was 7%. In comparative experiments the results are represented as relative membrane movement = $\frac{\text{movement experimental (+ drug)}}{\text{movement control (- drug)}} \times 100$. The dose response of HeLa cells (naive) and tolerant HeLa cells (415 days) is shown in Figure 8. Note that the concentration of morphine which decreases membrane movement by 50% is the same concentration which inhibits plating by 50%. The trained HeLa cells also show a tolerance to the inhibition of membrane movement, although not as great a tolerance as is seen in the plating assay. When morphine is removed from the medium, the membrane movement returns to its original value within 18 min.

Effect of Drugs on Attachment

For HeLa cells to form a colony when plated, two steps are required. These are: 1) attachment to the surface of the petri dish and 2) growth and division. The overall plating would show inhibition if either of these steps was inhibited by a given drug. To differentiate between these two possibilities, the following procedure was developed. HeLa cells (1×10^3) were plated into petri dishes containing medium 199 + 10% calf serum + 1.5 RP_{50} of drug. At various times, medium was removed, the plate washed with Hanks BSS, and 5.0 ml of 199 + 10% calf serum added. Cells are incubated, washed, stained, and counted as described in the materials and methods section. Virtually all the cells can be removed at 2 minutes, and attachment is essentially complete at 3 hours (97.8% of cells attached). The time course for HeLa attachment is shown in Figure 9. The effect of drugs on attachment is

Figure 8. Dose Response of Membrane Movement to Morphine Concentration

The membrane movement was determined as described in text. The movement in the absence of morphine is taken as the 100% value and the movements determined in the presence of the various drug concentrations expressed relative to its value. The experiment was initiated without morphine and after 45 minutes of filming, medium containing morphine at the appropriate concentration was added and filming resumed. Morphine concentration was increased at 45 minute intervals, flushing chamber between intervals. Control HeLa (0) and trained HeLa (●) cells are shown.

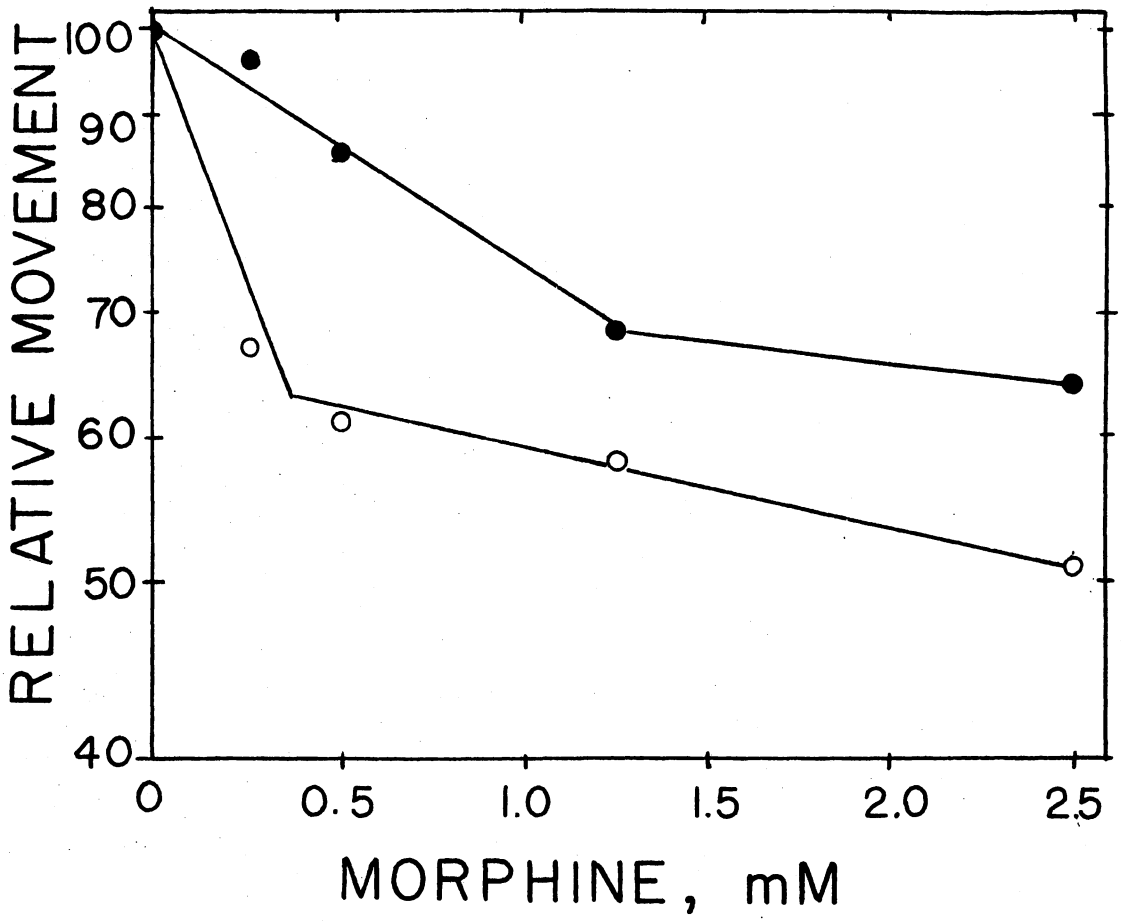
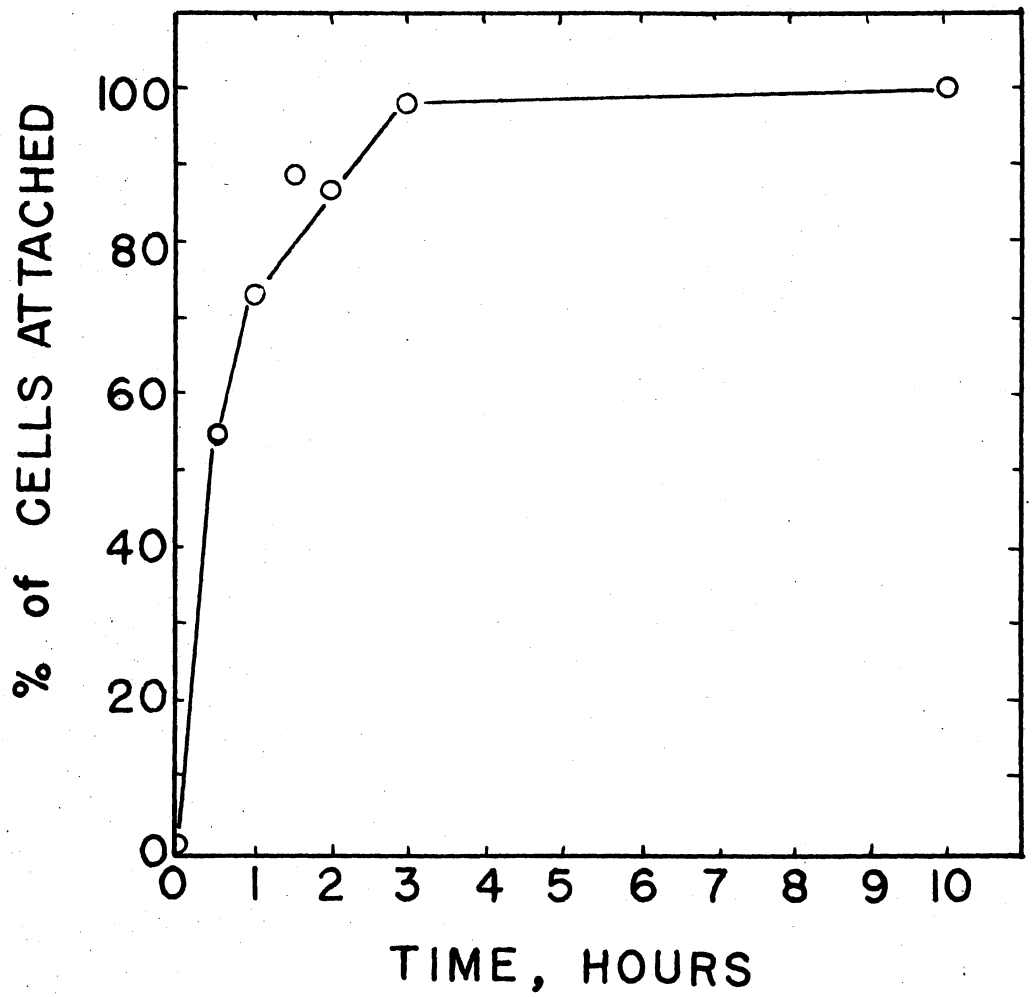

RELATIVE MOVEMENT
100
90
80
70
60
50
40
0
0.5
1.0
1.5
2.0
2.5
MORPHINE, mM

Figure 9. Time Course of HeLa Attachment

1×10^3 HeLa cells are plated/petri dish. Each point is the average of 4 experiments with triplicate determinations in each experiment.


% of CELLS ATTACHED
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
TIME, HOURS

shown in Table VII. Part A shows the drugs which inhibit attachment, part B shows the drugs that have no effect on attachment, and part C shows the control (for parts A and B).

Withdrawal of Drug from Tolerant Cells

Addiction requires that cells develop a requirement for the drug for growth along with increased tolerance. A measure of addiction in cell culture is that the growth rate of addicted cells should be slower and/or these cells should have a reduced plating efficiency when the drug is removed from the medium. These effects should be reversible on restoration of the drug.

To determine the effects of withdrawal on tolerant cells, the cell number and plating efficiency were determined after growing trained HeLa cells in the presence and absence of drug. The growth of untrained HeLa's in the absence of drug is also measured. The protocol for these experiments is shown in Table VIII. Table IX shows the results from experiment A (two separate experimental conditions). Under the first condition, the tolerant HeLa cells are grown with no drug or with 0.5 RP_{50} drug concentration (A-1, 12 d withdrawal) and under the second condition with no drug and 1.0 RP_{50} drug concentration (A-2, 13 d withdrawal). Amphetamine, diphenhydramine hydrochloride (benadryl) and morphine (with the exception of experiment A-2 for diphenhydramine hydrochloride) show a higher cell number for tolerant cells grown in the presence of drug than in its absence.

After the 12 day withdrawal period the cells from experiment A-1 were inoculated into MDB (Experiment B) or were plated on petri dishes (Experiment C).

TABLE VII

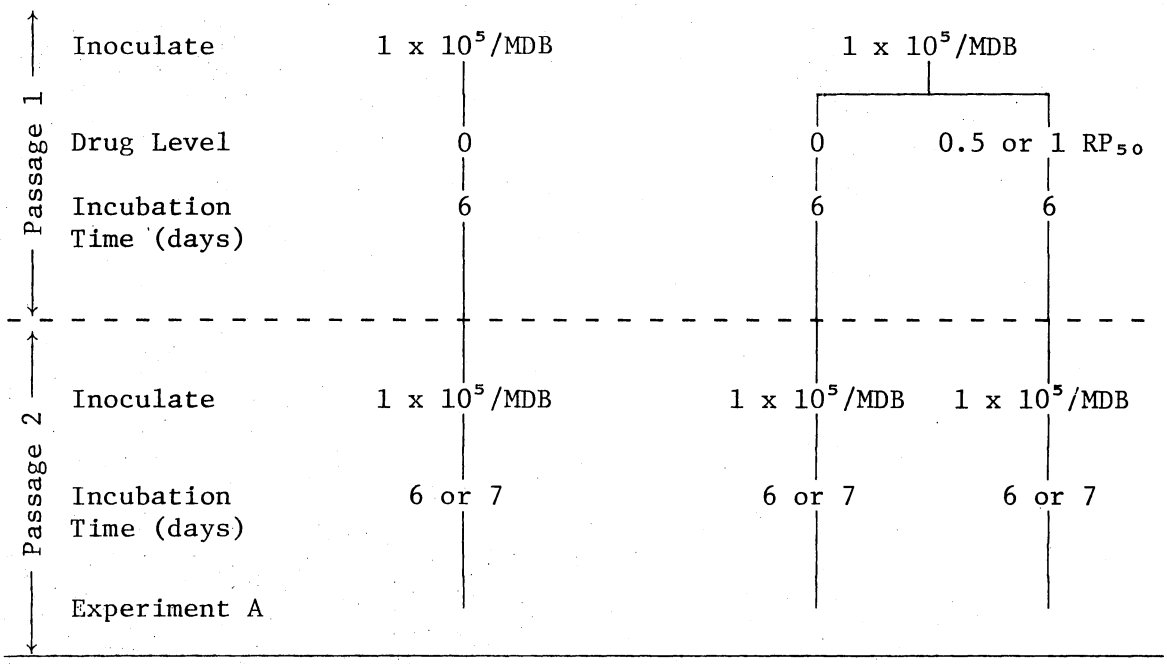
EFFECT OF DRUGS ON ATTACHMENT

Drug	Number of Cells Attached at 1 Hour	3 Hours
A. Inhibitory		
Amphetamine	111	213
Caffeine	59	135
Diazepam	145	227
Dimethylsulfoxide	53	118
Phenobarbitol	103	175
Propoxyphene	78	190
B. No Effect		
Acetylsalicylic acid	215	289
Codeine	197	276
Diphenhydramine HCl	158	271
Ethanol	190	266
Meprobamate	140	277
Morphine	157	261
Phenethylalcohol	172	239
Thorazine	205	293
C. Control		
No addition	191	258

HeLa cells (1×10^3) are plated and grown as described in Materials and Methods. After 7 days, plates are washed and stained. Number of colonies on plate = number of cells attached.

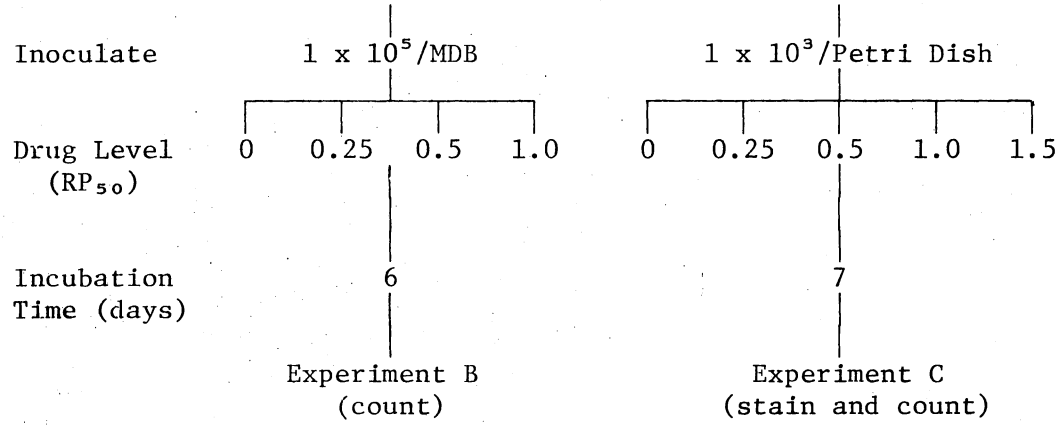
TABLE VIII

WITHDRAWAL PROTOCOL



The cells in the MDB's were removed by trypsinization and appropriate aliquots counted using the Coulter Counter to determine the number of cells per bottle. For Experiment A-1 the count was made after 6 days of incubation, and in Experiment A-2 after 7 days of incubation.

Cells from Experiment A-1 treated as follows:



The relative growth in experiment B is obtained by comparing the cell yield obtained in the presence of drug with that of aliquots from the same inoculum grown in the absence of drugs. The total withdrawal time was 18 days (12 + 6). With both diphenhydramine hydrochloride and morphine tolerant cells, the relative growth of the cells was increased by low concentrations of the drug. This is shown in Table X.

Table XI shows the results from experiment C. The relative plating efficiency is obtained by comparing the number of colonies obtained in the presence of the drug with the colonies in the absence of the drug. Total withdrawal time for cells in this experiment is 19 days (12 + 7). Amphetamine and diphenhydramine hydrochloride trained cells showed better plating in the presence of low concentration of the drug than in its absence. The morphine tolerant cells, which showed better growth in MDB in presence of morphine did not show the same result in the plating assay. The disparity between the amphetamine and morphine results in the MDB growth experiment and the plating assay suggests different parameters contribute to the end measurement of growth and that the influence of cultivation in the presence of drug addiction in these parameters may be diverse enough to yield different responses.

Morphologic Observations

When drugs are added to control HeLa cells there is an increase in the number of vacuoles and cells "round up". These effects are not seen in the trained cell lines. However, the morphology of individual cells and colonies is altered during the training process.

TABLE IX

EFFECT OF CULTIVATION IN ABSENCE OF DRUG ON GROWTH OF CELLS PREVIOUSLY GROWN IN RP_{50} DRUG CONCENTRATIONS

Experiment A			
	Drug During Growth (RP_{50})	Cell Number x 10^{-5}	
		Tolerant Cells	Control Cells
Amphetamine			
1	0 (withdrawn 12 days)	5.5	7.9
	0.5	6.8	
2	0 (withdrawn 13 days)	9.8	10.6
	1.0	10.0	
Diphenhydramine hydrochloride			
1	0 (withdrawn 12 days)	3.6	7.8
	0.5	5.8	
2	0 (withdrawn 13 days)	11.8	12.6
	1.0	10.3	
Morphine			
1	0 (withdrawn 12 days)	4.4	7.8
	0.5	5.2	
2	0 (withdrawn 13 days)	4.5	4.7
	1.0	5.2	

Trained or untrained (control) HeLa cells treated according to protocol in Table VIII. Cell number is determined by trypsinization to remove cells from MDB and counting on a Coulter Counter.

TABLE X

EFFECT OF DRUG CONCENTRATION ON RELATIVE GROWTH OF WITHDRAWN, MAINTAINED (0.5 RP_{50}), AND UNTREATED HELA CELLS

Experiment B Drug Cell History Growth Condition	Relative Growth Drug Concentration x RP_{50} 0.25	0.5	1.0
Amphetamine			
Tolerant HeLa			
0 (withdrawn 12 days)	91	91	42
0.5	110	86	37
Untreated HeLa	73	48	17
Diphenhydramine hydrochloride			
Tolerant HeLa			
0 (withdrawn 12 days)	140	124	53
0.5	125	84	55
Untreated HeLa	90	43	27
Morphine			
Tolerant HeLa			
0 (withdrawn 12 days)	106	113	71
0.5	111	115	88
Untreated HeLa	104	66	45

$$\text{Relative Growth} = \frac{\text{Growth in presence of drug}}{\text{Growth in absence of drug}} \times 100$$

Tolerant or control HeLa cells treated according to protocol in Table VIII. Total withdrawal time for HeLa was 18 days.

TABLE XI

EFFECT OF DRUG CONCENTRATION ON RELATIVE PLATING EFFICIENCY OF WITHDRAWN, MAINTAINED (0.5 RP_{50}), AND UNTREATED HELA CELLS

Experiment C Drug Cell History Growth Conditions	Relative Plating Efficiency Drug Concentration x RP_{50} 0.25	0.5	1.0	1.5
Amphetamine				
Tolerant HeLa				
0 (withdrawn 12 days)	117	115	120	98
0.5	102	106	105	102
Untreated HeLa	89	80	31	16
Diphenhydramine hydrochloride				
Tolerant HeLa				
0 (withdrawn 12 days)	112	119	121	89
0.5	102	102	116	107
Untreated HeLa	86	55	35	4
Morphine				
Tolerant HeLa				
0 (withdrawn 12 days)	97	90	87	74
0.5	99	85	89	70
Untreated HeLa	97	93	35	1.3

$$\text{Relative Plating Efficiency} = \frac{\text{Colonies in presence of drug}}{\text{Colonies in absence of drug}} \times 100$$

Tolerant or control HeLa cells treated according to protocol in Table VIII. Total withdrawal time for trained HeLa is 19 days.

There were numerous elongated cells in loose, irregular colonies when the cells were grown in the presence of acetylsalicylic acid, amphetamine, meprobromate, morphine, propoxyphene, and phenobarbital. There was reversion to typical cell and colony types upon removal of morphine and phenobarbital, but not with the other drugs. With diphenhydramine hydrochloride there were single cells and small colonies with parallelly oriented cells that did not revert upon removal of the drug. Parallel oriented elongated cells with the cells projecting out from the colony were produced by growth in the presence of codeine and these cells did not revert upon removal of codeine. Diazepam produced elongated single cells which did not revert.

Binding of [^{3}H]-Morphine

The observations of the effect of morphine on the membrane motion, the decreased effect of morphine on the membrane motion of tolerant cells, and the knowledge of the existence of specific opiate receptors on the plasma membrane led to studies on the binding of [^{3}H]-morphine to HeLa cells.

The time course for binding of [^{3}H]-morphine is shown in Figure 10. In this experiment, 2×10^6 HeLa cells are incubated in buffer (0.01 M Tris, 0.32 M sucrose, pH 8.0) containing 1.35×10^5 CPM of [^{3}H]-morphine (morphine concentration 1×10^{-9} M). Cells are collected, rinsed, dried, and radioactivity determined as in materials and methods. Binding is maximal at 30 min.

The binding of [^{3}H]-morphine to HeLa cells as a function of morphine concentration is shown in Figure 11. The morphine tolerant HeLa cells used in the binding experiments were trained HeLa cells

Figure 10. Time Course of Binding of [^{3}H]-Morphine by HeLa Cells.

HeLa cells are incubated (at 37°) with [^{3}H]-morphine (135,000 CPM, 10^{-9} M morphine) in 0.01 M Tris, 0.32 M sucrose (76). Cells are collected in GF/B filter after indicated time (vacuum aspiration), rinsed, air dried, and binding of [^{3}H]-morphine determined on a Packard Tri Carb in Bray's (174) cocktail. Each point is an average of triplicate determinations.

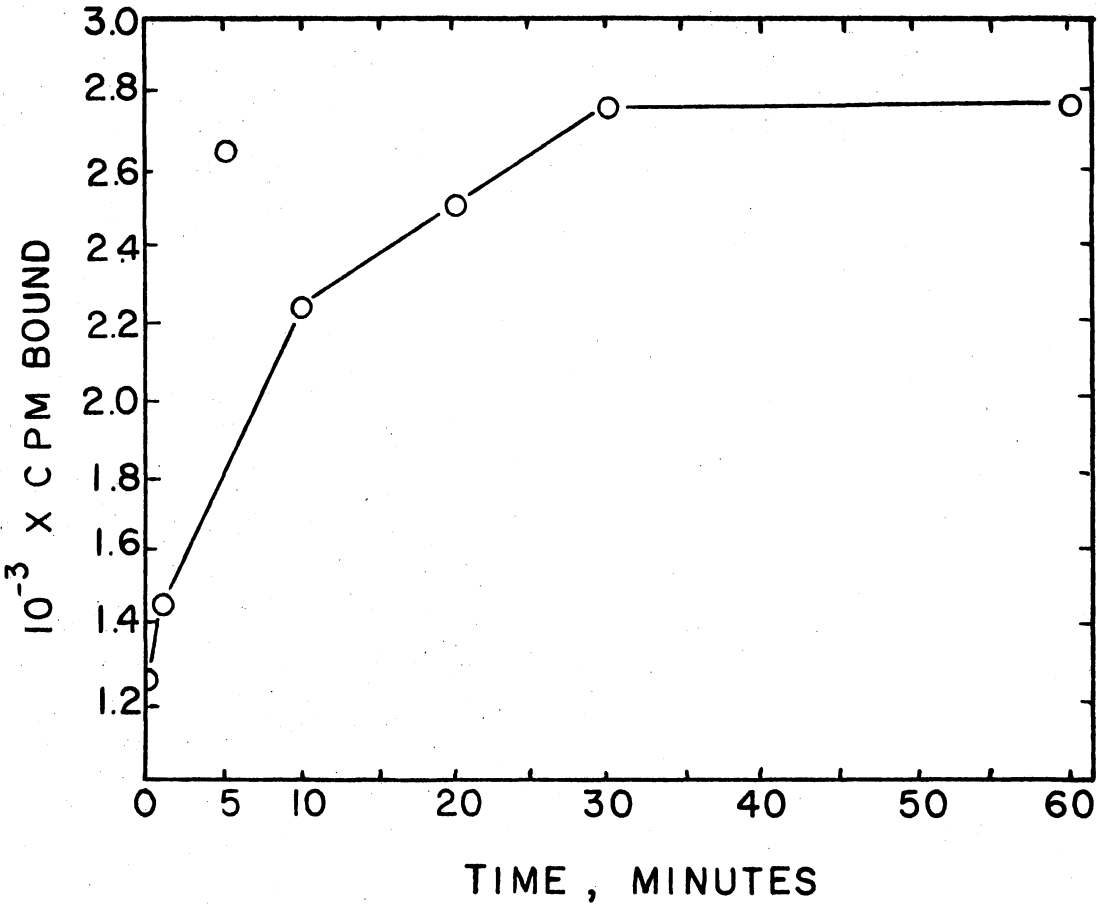
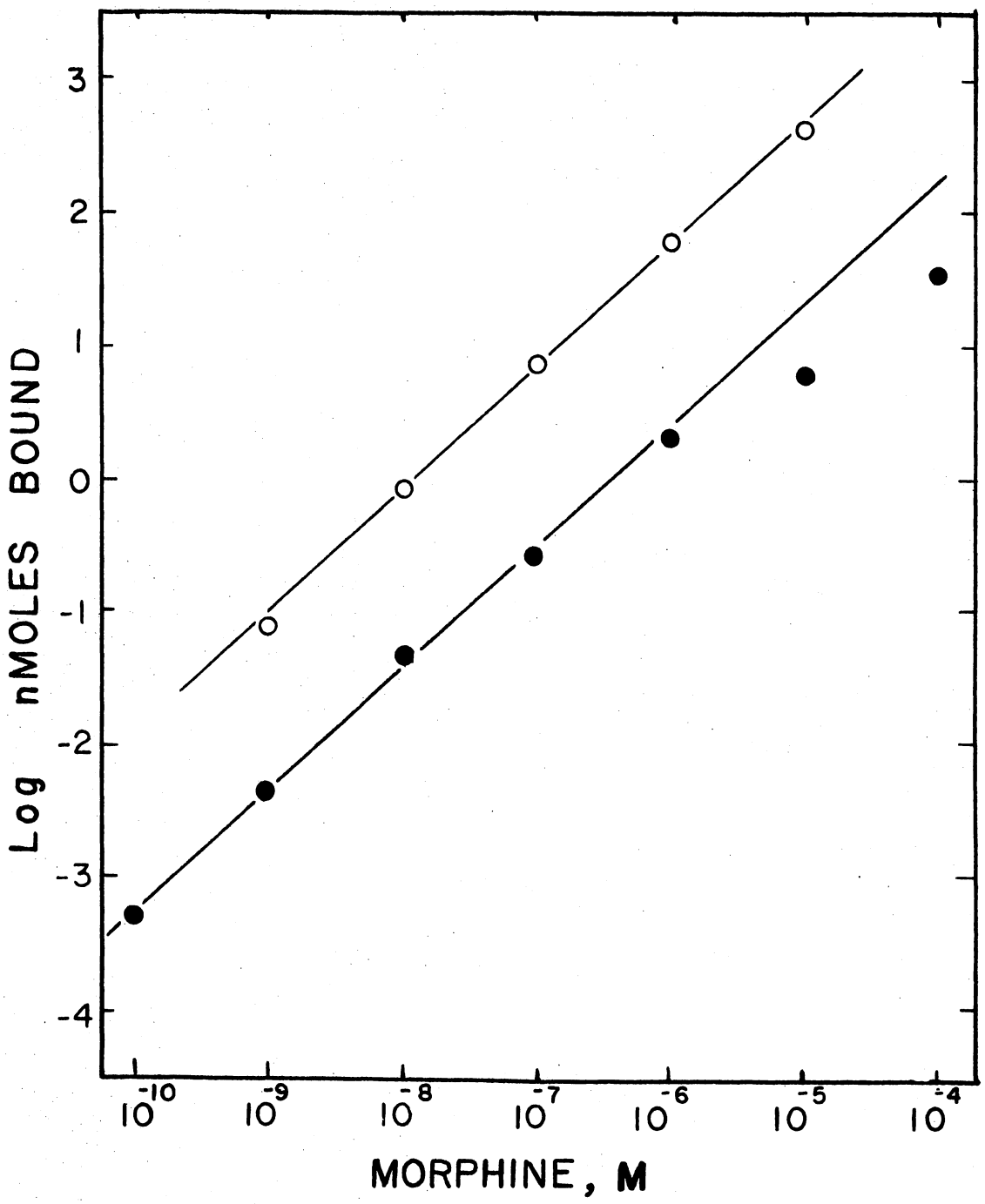

3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
10^{-3} X CPM BOUND
0
5
10
20
30
40
50
60
TIME , MINUTES

Figure 11. nMoles Morphine Bound Versus Morphine Concentration.

1.5×10^6 tolerant HeLa (0) or control HeLa (●) cells are incubated (at 37°) with 1.58×10^5 CPM of [^{3}H]-morphine in Tris-sucrose buffer containing indicated morphine concentration. CPM bound after 30 min is used to determine nM bound.


Log nMOLES BOUND
3
2
1
0
-1
-2
-3
-4
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
MORPHINE, M

frozen at 645 days, stored for 1 year, re-initiated, and maintained at 1.0 RP_{50} morphine for 450 days. When plated, these cells do not show increased tolerance to morphine. However, they can grow well in 1.0 RP_{50} morphine whereas the control HeLa cells die within 3 passages.

In this experiment, 1.5×10^6 cells were incubated with [^{3}H]-morphine (1.58×10^5 CPM) in 0.01 M Tris, 0.32 M sucrose buffer (pH 8.0) containing indicated morphine concentration for 30 minutes, collected, rinsed, dried and counted as in methods. The amount bound was calculated by comparing CPM bound with CPM/nmole. There is more morphine bound by trained cells than by non-trained cells, but in neither case does the binding show saturability which would be expected from an opiate receptor.

The binding of [^{3}H]-opiate to the receptor should be stereo-specific as shown by Goldstein et al. (86). Using Pert and Snyder's (87) rapid filtration method, binding of [^{3}H]-morphine to HeLa cells (trained and control) and purified plasma membranes of HeLa (trained and control) was determined in the presence of morphine (10^{-9} M) or morphine (10^{-9} M) + dextrorphan or levorphanol (10^{-8} M). Plasma membranes were purified as described in Materials and Methods. The membranes show a 13 fold increase in plasma membrane markers; a small contamination with endoplasmic reticulum and mitochondria; with no nuclear contamination observed (visual monitoring during preparation). Table XII shows the effects of 10^{-8} M levorphanol or dextrorphan on the binding of [^{3}H]-morphine (1.5×10^5 CPM) on HeLa cells (tolerant or control) or their purified plasma membranes in 0.01 M Tris, 0.32 M sucrose buffer (pH 8.0). Dextrorphan and levorphanol decrease binding by about the same amount in trained and untrained HeLa cells, with no

TABLE XII

EFFECT OF DEXTRORPHAN AND LEVORPHANOL ON
[^{3}H]-MORPHINE BINDING

Preparation	CPM Bound		
	Control	Dextrorphan	Levorphanol
A. Cells			
HeLa	2348	944	861
Trained HeLa	11441	5211	5465
B. Plasma Membrane			
HeLa	337	303	457
Trained HeLa	353	247	177

A. 4×10^6 HeLa (tolerant or control) cells incubated (30 min) with 1.5×10^6 CPM of [^{3}H]-morphine in buffer (0.01 M Tris, 0.32 M sucrose, pH 8) at 37° containing either 10^{-9} M morphine or 10^{-9} M morphine + 10^{-8} M dextrorphan or levorphanol. Binding measured as in materials and methods.

B. 0.5 mg of purified plasma membrane from tolerant or control HeLa cells incubated in Tris-sucrose buffer at 37° containing 1.5×10^5 CPM [^{3}H]-morphine and morphine (10^{-9} M) or morphine (10^{-9} M) + dextrorphan or levorphanol (10^{-8} M). Binding measured as in materials and methods.

TABLE XIII

DISPLACEMENT OF BOUND [^{3}H]-MORPHINE BY VARIOUS DRUGS

Drug	% of Control Binding Remaining	
	1 minute	10 minutes
Acetylsalicylic acid	52.9 ± 11.1	36.0 ± 4.1
Amphetamine	85.1 ± 3.6	52.6 ± 12.1
Codeine	64.1 ± 6.8	46.6 ± 11.1
Dextrorphan	65.5 ± 13.2	51.2 ± 11.5
Levorphanol	76.2 ± 9.6	38.8 ± 3.3
Morphine	62.0 ± 8.6	30.6 ± 8.7
Phenobarbital	66.4 ± 21.5	46.5 ± 9.8
Procaine	77.8 ± 5.9	52.1 ± 3.4
Propoxyphene	88.9 ± 13.8	66.6 ± 4.4

HeLa cells were incubated with [^{3}H]-morphine for 30 min in 0.01 M Tris, 0.32 M sucrose, pH 8 containing 10^{-9} M morphine for 30 min at 37°. Cells are centrifuged (5,000 RPM), buffer decanted, and cells resuspended in Tris-sucrose buffer containing 10^{-8} M drug for indicated time. Cells are collected, washed, air dried, and counted as described in methods. Binding is compared to control treated in the same manner but collected immediately after re-suspension in buffer with drug.

discernable discrimination between the two. Binding to purified plasma membranes was too low to observe significant effects. Dextrorphan and levorphanol decreases the binding on membranes from tolerant cells, but had little effects on the membranes from control cells. This shows the binding is not specific, since the inactive D-isomer (dextrorphan) inhibits binding about the same as the active, L-isomer (levorphanol). Table XIII shows the displacement by various drugs of [^{3}H]-morphine bound to HeLa cells. All of the drugs tested displaced bound [^{3}H]-morphine with 10-50% displacement occuring in the first minute. This indicates that binding is non-specific and loose.

CHAPTER IV

GROWTH OF ANIMAL CELLS ON GLASS FIBER FILTERS AND THEIR UTILIZATION FOR BIOSYNTHETIC ANALYSIS

To assess the effect of drugs on transport and incorporation of radioactive precursors, several replicate cultures are required. This can readily be obtained in suspension culture by labelling the culture and using aliquots to determine the time course, effects of inhibitors, and other parameters. HeLa cells, however, do not rapidly adjust to suspension, and since toxicities and other work were done with cultures grown as monolayers, we desired to develop a simple system to assess the effect of drugs on the uptake and incorporation of labeled precursors.

Effect of Filter Type and Growth Support on Uptake

Several types of filters and growth supports were compared by measuring [^{3}H]-thymidine uptake by HeLa cells. Support systems (Millipore filter, Whatman GF/A filter, Reeves Angel Glass Fiber filter, Corning coverslip, and glass scintillation vials) were sterilized as in Materials and Methods and inoculated with 1 x 10^3 or 1 x 10^6 HeLa cells/support in 0.25 ml of medium 199 + 10% calf serum (1.0 ml final volume for scintillation vials). Cells are allowed to attach for 4 h,

medium volume brought up to 10.0 ml (5.0 ml for scintillation vial), and grown an additional 20 h (not required). Cells were then pulsed with [^{3}H]-TdR (0.5 μCi/ml) for 12.0 h. The pulse was terminated by removal of labeled medium and rinsing 2X with 10.0 ml of Hanks Balanced Salt Solution (173) and air dried. Uptake was determined as described in Materials and Methods. Table XIV shows the CPM of [^{3}H]-thymidine taken up by the cells grown on various supports. From this table, the superiority of the two glass fiber filters over the other supports can be seen. Whatman GF/A filters were chosen for further experiments.

To compare the uptake by cells grown on glass fiber filters to that by cells grown in MDB, HeLa cells (1×10^3 - 1×10^6) were plated on filters (0.25 ml) or MDB (2.0 ml), and grown as described in Materials and Methods. Cultures were pulsed for 4.0 h with [^{3}H]-thymidine (0.5 μCi/ml). Pulse was terminated and uptake determined as described in Materials and Methods. The CPM taken up by cells on the two different supports vs. cells plated/support is shown in Figure 12. A CPS program for linear regression gave the slopes of the lines of log CPM versus log cells plated/support as 0.842 ± 0.004 for the experiment done on MDB and 0.838 ± 0.002 for the experiment done on glass fiber filters. Cleland's application of the Student t test on the slopes (183) shows that there is no significant difference (t 0.2, 46). With 10^6 cells the glass fiber filter (calculated surface area, 4.52 cm^2) was saturated while the uptake by cells in the MDB (calculated surface area, 40 cm^2) was still linear with cell number.

Measurement of DNA, RNA, and Protein Synthesis

The synthesis of specific macromolecules can be measured by

TABLE XIV

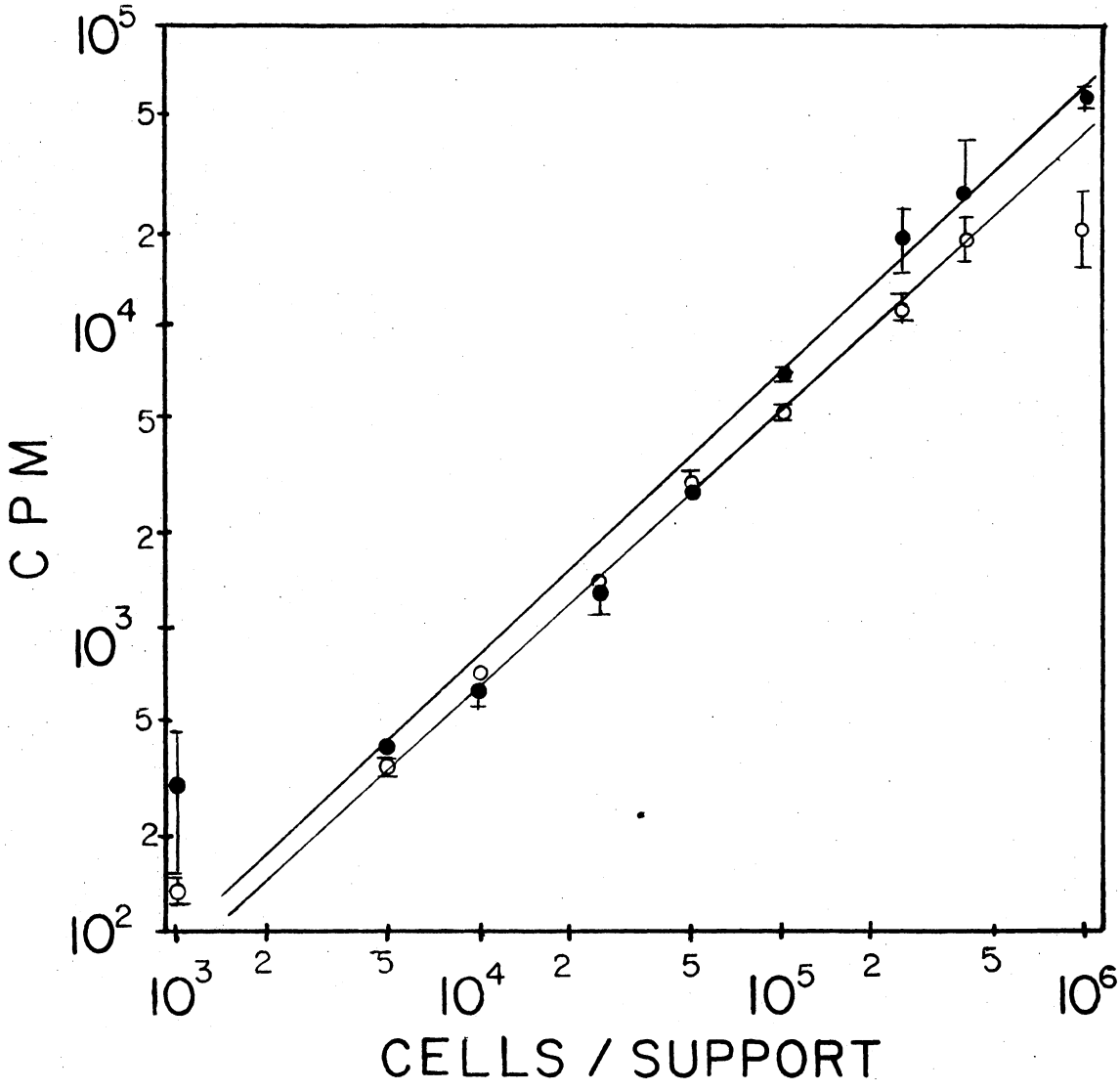
THE EFFECT OF FILTER TYPE OR SUPPORT ON [^{3}H]-THYMIDINE UPTAKE

Type of Filter or Support	Cells Plated	
	10^3	10^6
	CPM ± SD	
Millipore Filter	551 ± 94	39738 ± 5180
Whatman GF/A	4810 ± 586	212418 ± 8287
Reeves Angel Glass Fiber Filter	2714 ± 384	163457 ± 47590
Corning Coverslip	491 ± 59	46082 ± 8917
Scintillation Vials	1575 ± 531	97944 ± 15585

Triplicate samples of cells, either 10^3 or 10^6, were plated onto the indicated filter or support and incubated 4 h for attachment. Then 9.25 ml of medium 199 + 10% calf serum (4 ml for scintillation vials) was added to each petri dish and the contents incubated for 20 h. A 12 h pulse with 5 µCi of [^{3}H]-thymidine was made and the filters or supports were washed two times with 15 ml of Hank's Balanced Salts solution. Radioactivity was determined as described in the Materials and Methods section.

Figure 12. Comparison of Uptake by HeLa Cells Grown in MDB or on GF/A Filters

HeLa cells were plated on MDB (●) or GF/A filters (O) to give indicated number of cells/support. Cells were attached, grown, pulsed (4.0 h, 0.5 μCi [^{3}H]-TdR/ml), and counted as described in Materials and Methods.


10^5
5
2
10^4
5
2
10^3
5
2
10^2
CPM
10^3
2
5
10^4
2
5
10^5
2
5
10^6
CELLS / SUPPORT

determining the incorporation of specific radioactive precursors into acid insoluble material (156). [^{3}H]-Thymidine can be used to measure synthesis of DNA, [^{3}H]-uridine to measure synthesis of RNA, and [^{3}H]-tyrosine to measure the synthesis of protein. The difference between uptake and incorporation represents the amount transported into the intracellular pool (152).

The uptake and/or incorporation of radioactive precursor should show proportionality to cell number, concentration of precursor, and duration of pulse. Figure 13 shows these proportionalities for uptake of [^{3}H]-thymidine by HeLa cells. Uptake and incorporation of [^{3}H]-thymidine was linear with respect to cell number and concentration of [^{3}H]-thymidine. Uptake of [^{3}H]-thymidine was linear with respect to duration of pulse (incorporation was not measured). Figure 14 shows the proportionalities for uptake and incorporation of [^{3}H]-uridine by HeLa cells. Uptake and incorporation of [^{3}H]-UdR is linear with respect to number of cells, concentration of [^{3}H]-UdR, and duration of pulse. Figure 15 shows these proportionalities for uptake and incorporation of [^{3}H]-tyrosine by HeLa cells. Uptake and incorporation of [^{3}H]-tyr by HeLa cells is linear with respect to cells plated/filter and concentration of label. The uptake and incorporation of [^{3}H]-tyr is linear with respect to duration for the longer time points, but shows more error at shorter times. One of the reasons for the low incorporation and higher errors when [^{3}H]-tyr is used is that medium 199 has a high tyrosine content and the [^{3}H]-tyr used had a low specific activity (0.43 Ci/m mole). When LM cells are used in place of HeLa cells, uptake and incorporation of [^{3}H]-TdR is linear with respect to cells plated/filter. This can be seen in Figure 16.

Figure 13. Uptake and Incorporation of [^{3}H]-Thymidine by HeLa Cells

A. Uptake (●) and incorporation (O) of [^{3}H]-TdR versus cells/filter. Indicated number of cells are plated/filter, attached, grown, and pulsed (4.0 h, 0.5 μCi/ml) as described in Methods.

B. Uptake (●) and incorporation (O) of [^{3}H]-TdR versus concentration of [^{3}H]-TdR. HeLa cells (1 x 10^5) are plated, attached, grown, and pulsed for 4.0 h with indicated concentration of [^{3}H]-TdR.

C. Uptake of [^{3}H]-TdR versus duration of pulse. HeLa cells (1 x 10^5 filters) are plated, attached, and grown as described in Methods. Cells are pulsed with 0.5 μCi/ml for indicated duration.

Each point is average of triplicate samples. The error bars show the standard deviations of the measurement.

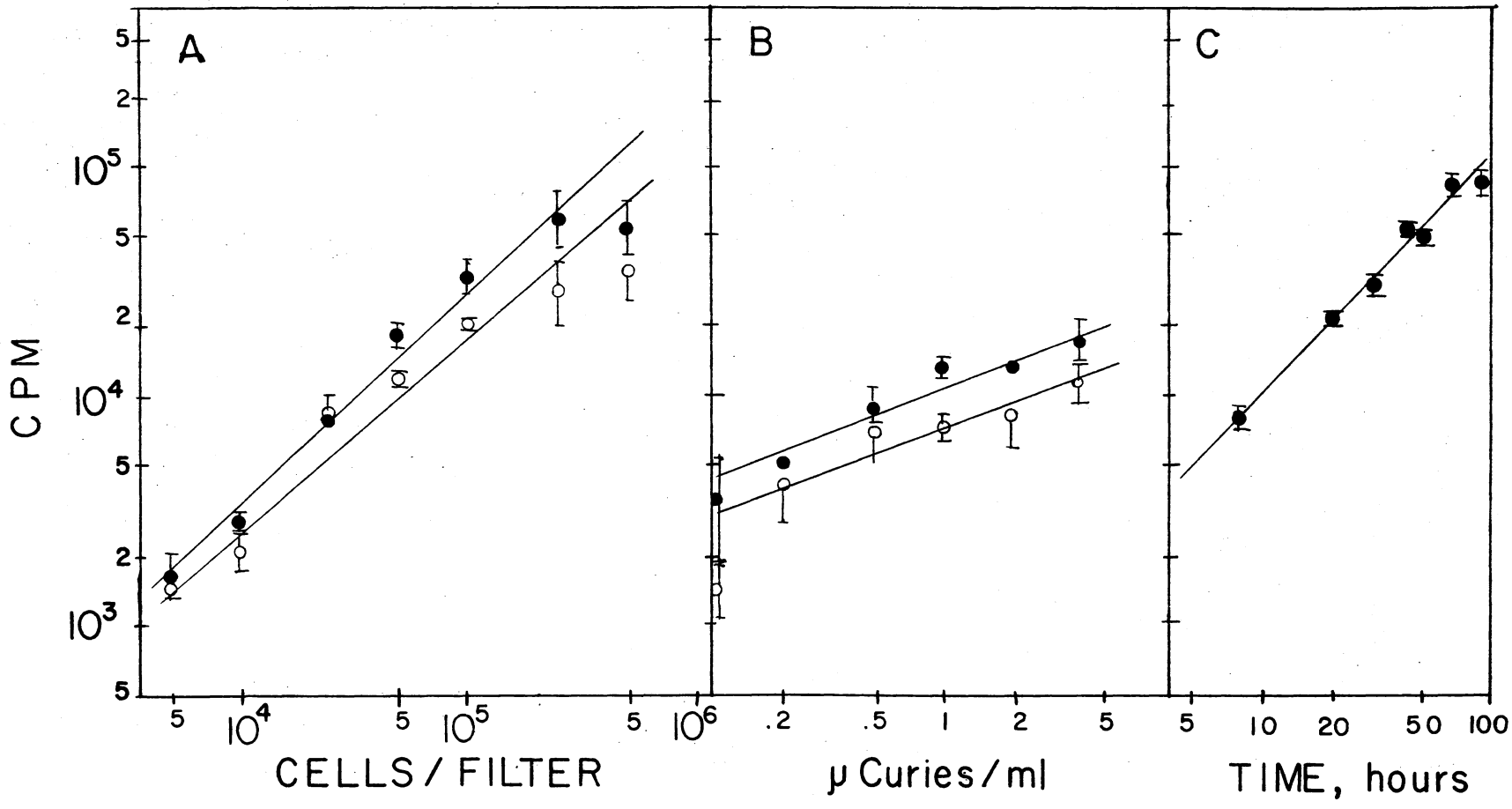

A
B
C
CPM
5
2
10^5
5
2
10^4
5
2
10^3
5
5
10^4
5
10^5
5
10^6
CELLS / FILTER
.2
.5
1
2
5
μ Curies / ml
5
10
20
50
100
TIME, hours

Figure 14. Uptake and Incorporation of [^{3}H]-Uridine by HeLa Cells

A. Uptake (●) and incorporation (O) of [^{3}H]-UdR versus cells/filter. Indicated number of cells are plated/filter, attached, grown, and pulsed (4 h, 0.5 μCi/ml) as described in Materials and Methods.

B. Uptake (●) and incorporation (O) of [^{3}H]-UdR versus concentration of [^{3}H]-UdR. HeLa cells (1 x 10^5) are plated, attached, grown, and pulsed for 4.0 h with indicated concentration of [^{3}H]-UdR.

C. Uptake (●) and incorporation (O) of [^{3}H]-UdR versus duration of pulse. HeLa cells (1 x 10^5 filter) are plated, attached, and grown as described in Methods. Cells are pulsed with 0.5 μCi [^{3}H]-UdR/ml for indicated duration.

Each point is average of triplicate samples. The error bars show the standard deviation of the measurement.

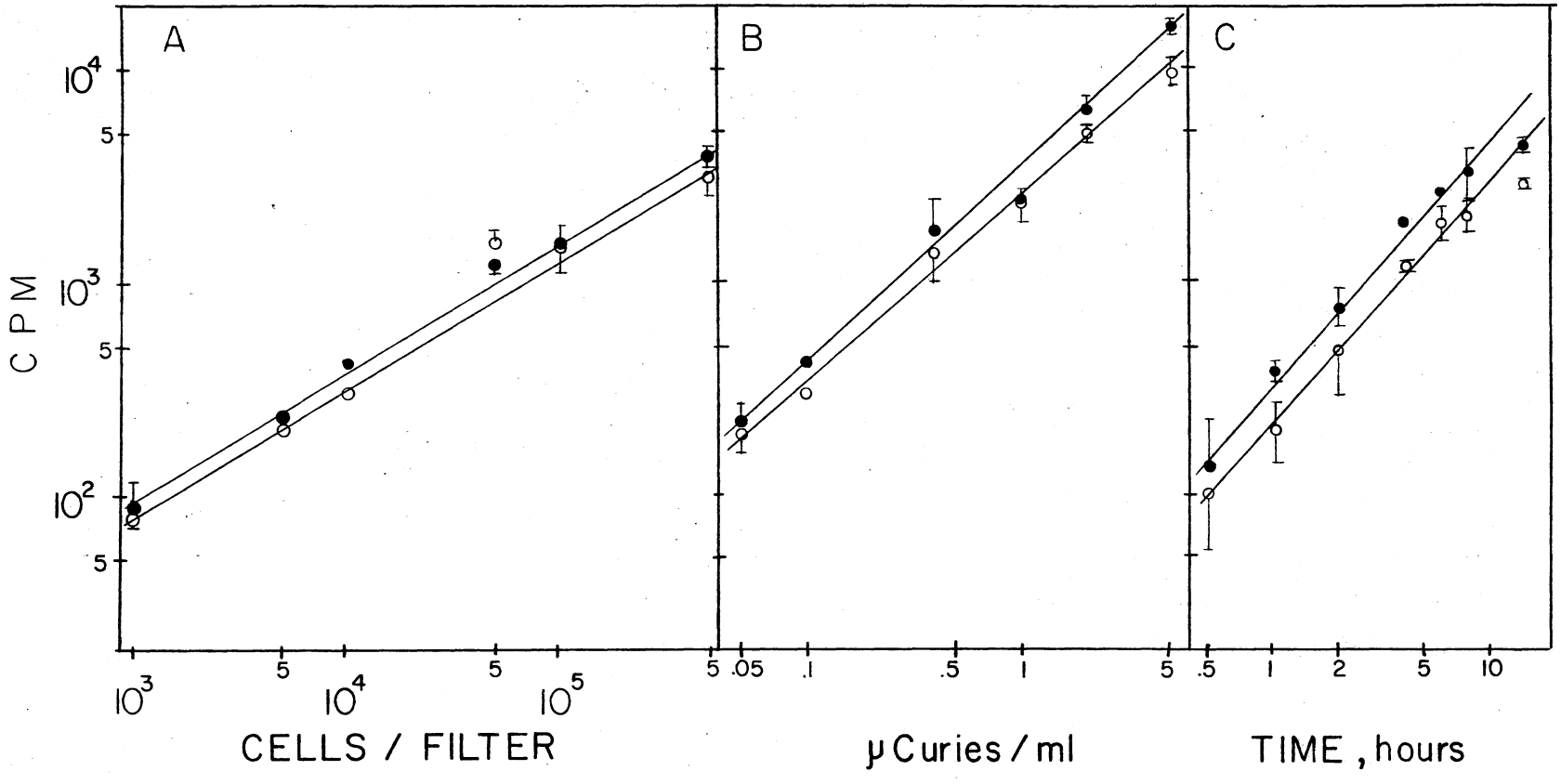

A
B
C
CPM
10^4
5
10^3
5
10^2
5
10^3
5
10^4
5
10^5
5
.05
.1
.5
1
5
.5
1
2
5
10
CELLS / FILTER
μCuries / ml
TIME , hours

Figure 15. Uptake and Incorporation of [^{3}H]-Tyrosine by HeLa Cells

A. Uptake (●) and incorporation (O) of [^{3}H]-Tyr versus cells/filter. Indicated number of HeLa cells are plated/filter, attached, grown and pulsed (4 h, 0.5 μCi/ml) as described in Materials and Methods.

B. Uptake (●) and incorporation (O) of [^{3}H]-Tyr versus concentration of [^{3}H]-Tyr. HeLa cells (1 x 10^5) are plated, attached, grown, and pulsed for 4.0 h with indicated concentration of [^{3}H]-Tyr.

C. Uptake (●) and incorporation (O) of [^{3}H]-tyrosine versus duration of pulse. HeLa cells (1 x 10^5/ filter) are plated, attached, and grown as described in Materials and Methods. Cells are pulsed with 0.5 μCi of [^{3}H]-Tyr/ml for indicated duration.

Each point is average of triplicate samples. The error bars show the standard deviation of the measurement.

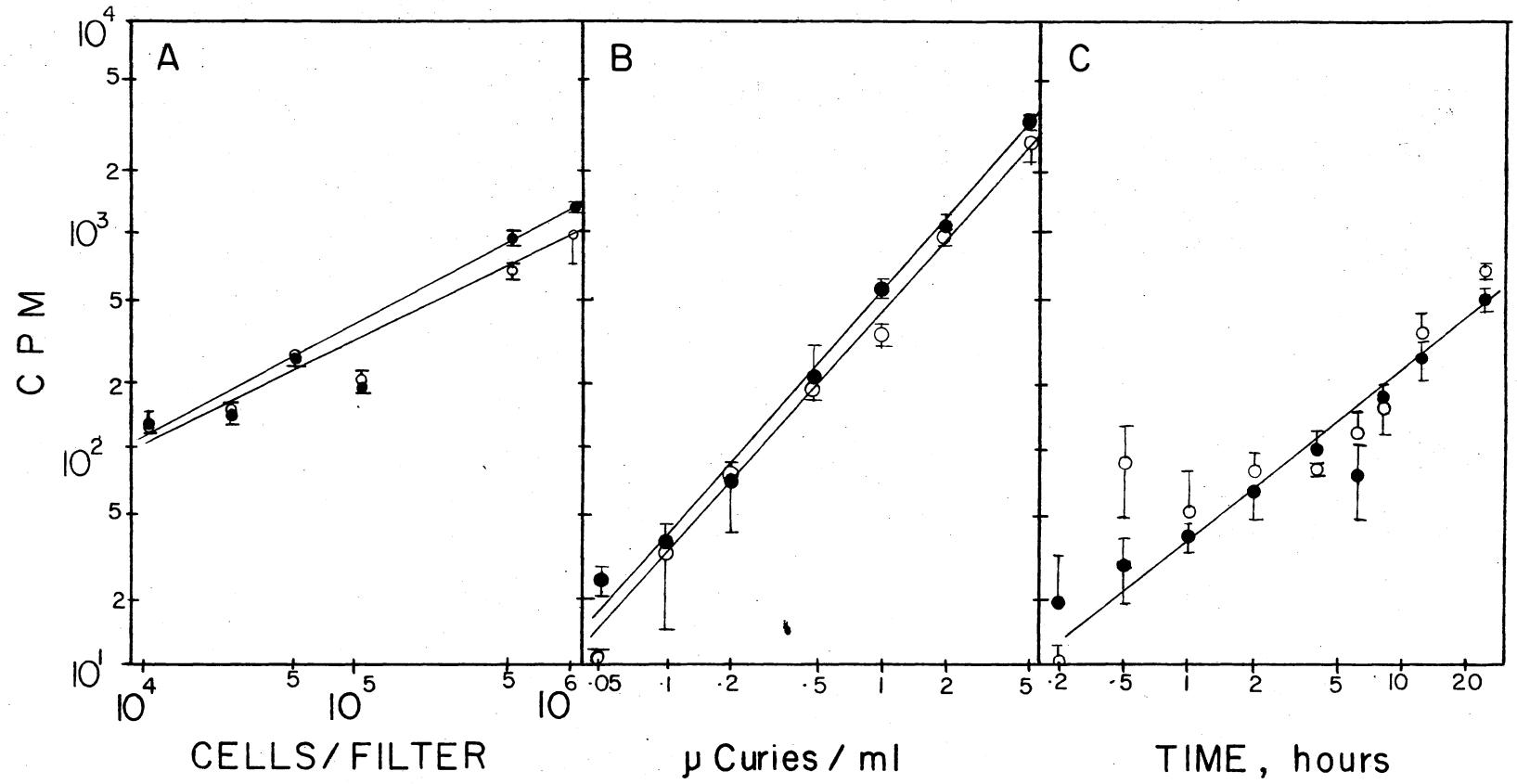
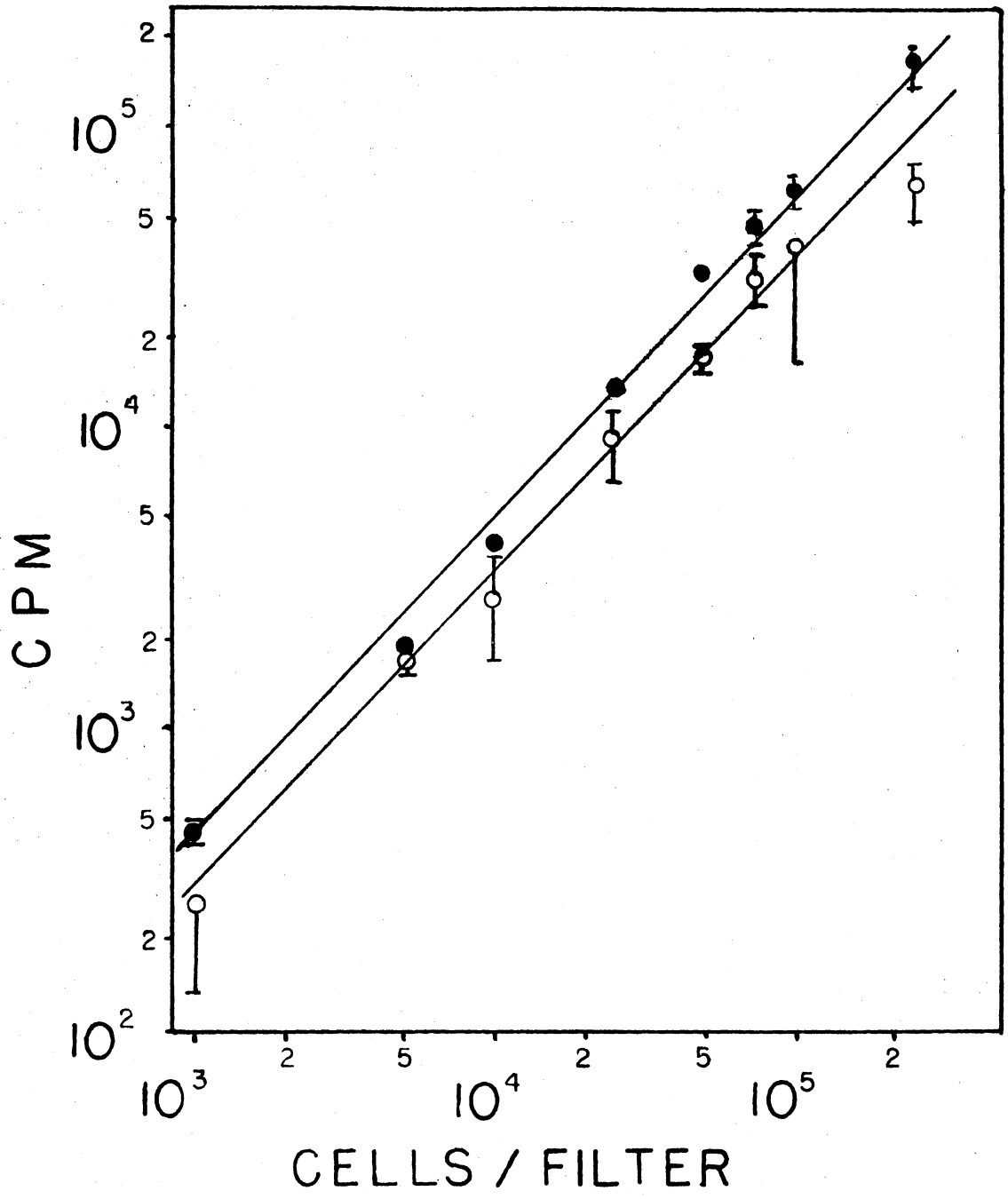

A
B
C
CPM
CELLS/FILTER
μ Curies / ml
TIME, hours

Figure 16. Uptake and Incorporation of [^{3}H]-Thymidine by LM Cells

Uptake (●) and incorporation (O) of [^{3}H]-TdR versus LM cells plated/filter. Indicated number of LM cells are plated/filter, attached, grown, and pulsed (4.0 h, 0.5 μCi/ml) as indicated in materials and methods.


CPM
2
10^5
5
2
10^4
5
2
10^3
5
2
10^2
10^3
2
5
10^4
2
5
10^5
2
CELLS / FILTER

Inhibition of [^{3}H]-Thymidine Uptake and Incorporation

Hydroxyurea inhibits the uptake and incorporation of thymidine and is a commonly used inhibitor of DNA synthesis (161). Figure 17 shows the effect of various concentrations of hydroxyurea on the uptake of [^{3}H]-thymidine. Thymidine incorporation shows the same dose response as does thymidine uptake as can be seen in Figure 18.

Morphine (0-2.0 RP_{50}) did not inhibit the uptake or incorporation of [^{3}H]-TdR by HeLa cells grown on glass fiber filters as can be seen in Table XV. These experiments were done with cells which failed to show the normal tolerance to morphine on plating, so inhibition of TdR uptake or incorporation cannot be ruled out as being involved in development of tolerance.

Figure 17. Hydroxyurea Inhibition of Thymidine Uptake

HeLa cells (10^5) were plated on GF/A filters, allowed to attach 4 h, and incubated 20 h. At 20 h, 2 ml of medium was removed and replaced by either fresh medium 199 + 10% calf serum or fresh medium 199 + 10% calf serum containing hydroxyurea to give the various final concentrations shown. [^{3}H]-Thymidine (0.5 µCi/ml) was added and the culture incubated for 4 h and the radioactivity determined as described in the Materials and Methods section. The plot shows the % of control value (7495 cpm taken up) versus hydroxyurea concentration. The error bars show the standard deviation of the triplicate measurements.

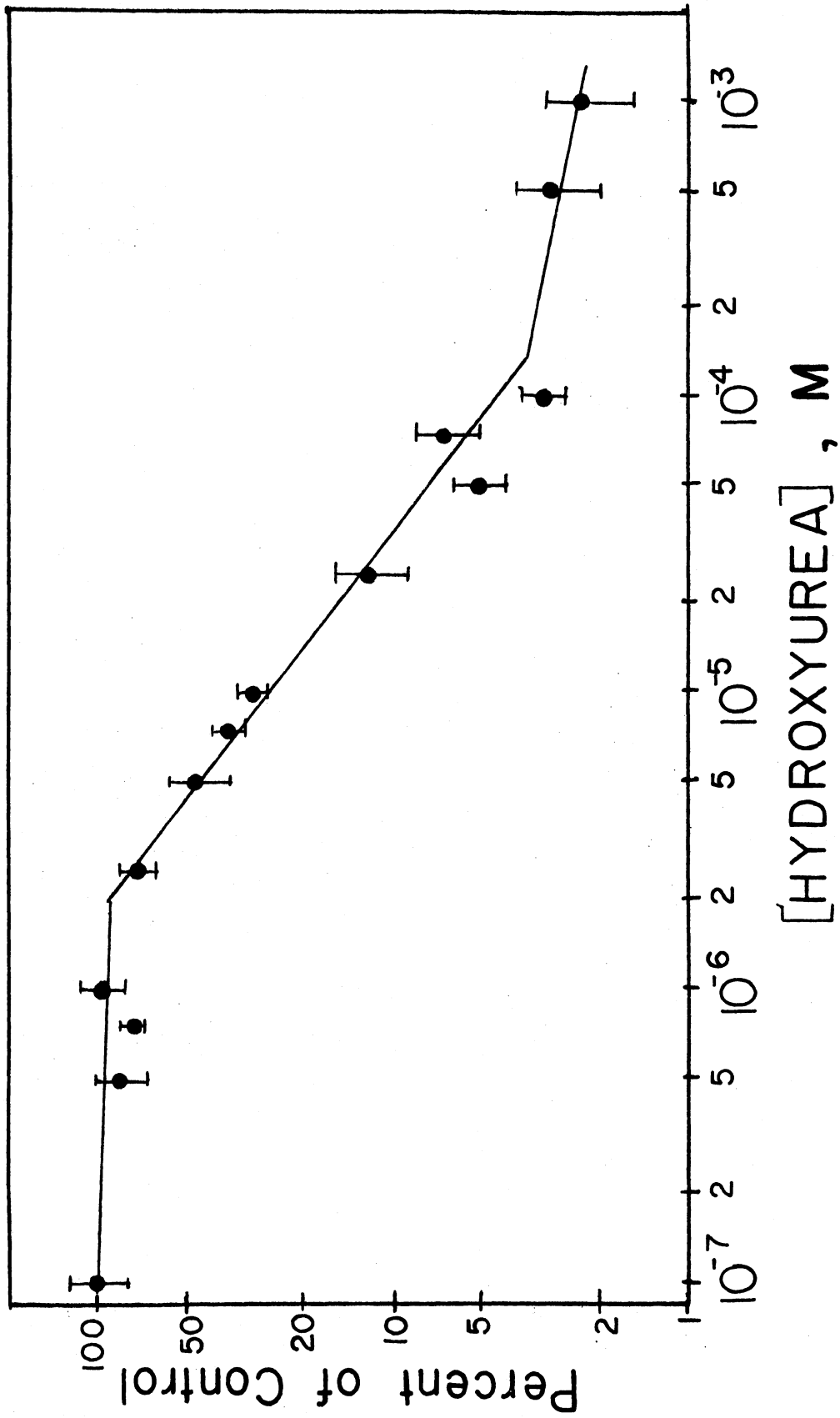

Percent of Control
100
50
20
10
5
2
1
10^{-7} 2 5 10^{-6} 2 5 10^{-5} 2 5 10^{-4} 2 5 10^{-3}
[HYDROXYUREA] , M

Figure 18. Hydroxyurea Inhibition of Thymidine Incorporation

HeLa cells (10^5) were plated on GF/A filters, allowed to attach 4 h, and incubated 20 h. At 20 h, 2 ml of medium was removed and replaced by either fresh medium 199 + 10% calf serum or fresh medium 199 + 10% calf serum containing hydroxyurea to give the various final concentrations shown. [^{3}H]-Thymidine (0.5 µCi/ml) was added and the culture incubated for 4 h and radioactivity determined as in the Materials and Methods section. The plot shows the % of control value (3462 CPM incorporated) versus hydroxyurea concentration. The error bars show the standard deviation of triplicate measurements.

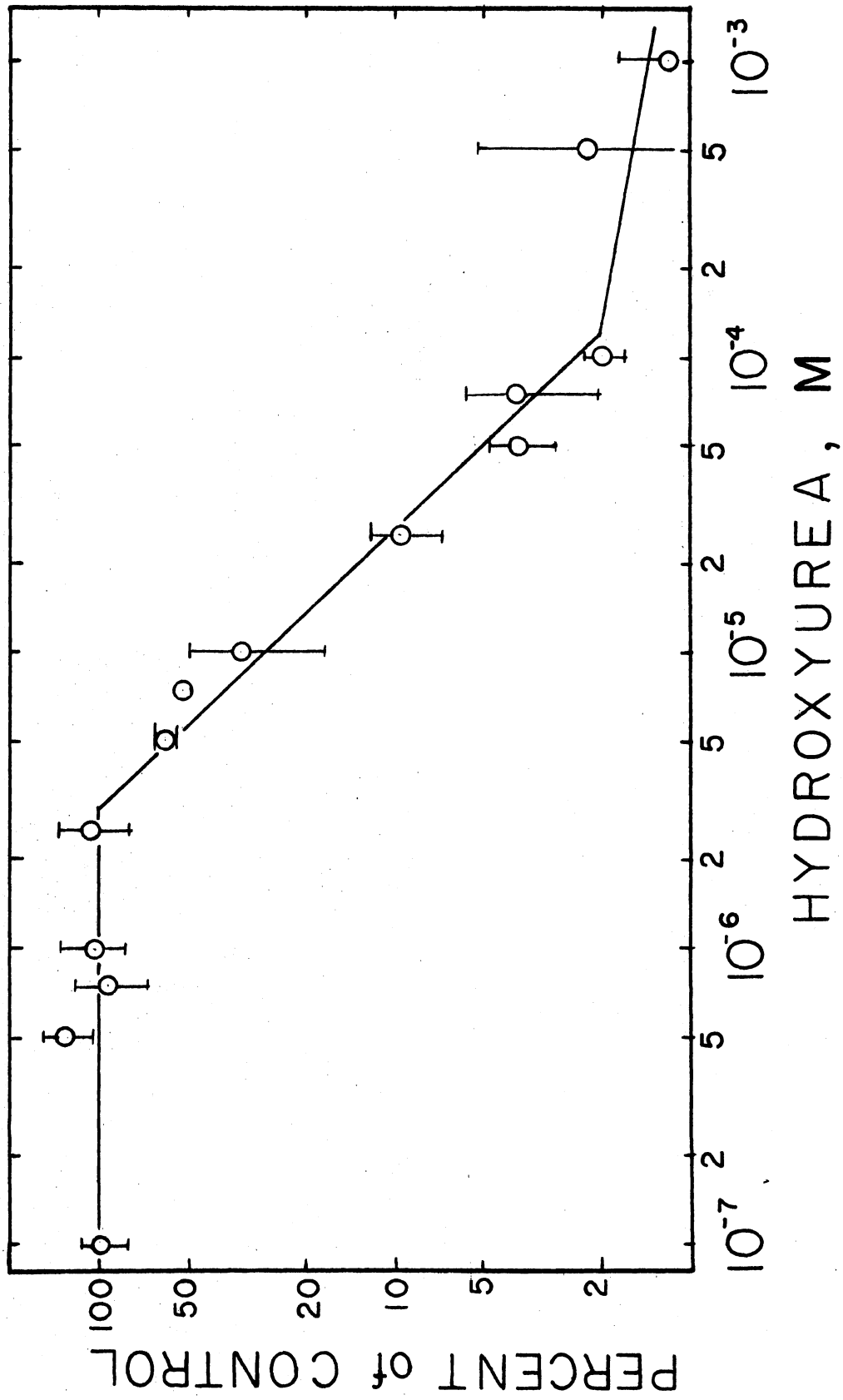

PERCENT of CONTROL
100
50
20
10
5
2
10^{-7} 2 5 10^{-6} 2 5 10^{-5} 2 5 10^{-4} 2 5 10^{-3}
HYDROXYUREA, M

TABLE XV

EFFECT OF MORPHINE ON UPTAKE OF [^{3}H]-THYMIDINE BY HeLa CELLS

Morphine Added mM	Uptake CPM ± SD	Incorporation CPM ± SD
0	4325 ± 742	2028 ± 187
0.63	4763 ± 857	2067 ± 271
1.25	4631 ± 761	2249 ± 182
2.50	4414 ± 772	2415 ± 384
3.75	4358 ± 886	1801 ± 238
5.00	4924 ± 1250	2304 ± 584

Duplicate samples of 10^5 cells were plated onto each of three GF/A filters, incubated 4 h for attachment, and then incubated 20 h in medium containing the appropriate concentration of morphine. At 20 h, 4 ml of medium was removed and replaced with fresh medium containing the appropriate concentration of morphine. [^{3}H]-Thymidine (0.5 µCi/ml) was added and the radioactivity determined as indicated in the Materials and Methods section after 4 h incubation.

CHAPTER V

DISCUSSION

Assessment of Toxicities of Drugs

Many of the prior investigators (described in the introduction) made qualitative observations to show that cells in culture show phenomena resembling tolerance and addiction. Sasaki (55) measured the diameter of explanted tissue fragments with a microscope projecting apparatus. Corssen and Skora (61) used time lapse cinematography and analyzed the results by slow projection, classifying growth as: growing well, growth retarded or arrested, and dead. Ghadirian (63) used microscopic observation of new born rabbit and puppy nervous tissue, classifying growth as: no growth, a few living cells, moderate growth and proliferation of cells, good growth with considerable migration, a stage intermediate between good growth and very good growth, and very good growth and migration. Heubner et al. (59) used the disappearance of pathological symptoms (fat globules and rounded cells) as a measure of tolerance.

One of the goals in this study was to quantitize tolerance, addiction, and withdrawal. A plating assay assessed the effect of various drugs on the colony forming ability of HeLa cells in culture. Dose responses of HeLa cells to morphine, codeine, and heroin are shown in Figure 2. The number of colonies formed in the presence of

drug is compared to the number formed in the absence of drug (relative plating efficiency). Table IV shows the effects of various drugs on HeLa plating. HeLa cells were more sensitive to heroin than to morphine (RP_{50} of 1.3 and 2.5 mM, respectively). HeLa cells are more sensitive to leucine enkephalin (RP_{50} 0.13 mM), which is the same relative sensitivity seen in whole animals. Codeine, which has only 1/10 the activity of morphine in vivo is more toxic to HeLa cells (RP_{50} of 1.0 mM versus 2.5 mM for morphine).

The concentration of morphine producing toxicity on HeLa cells corresponds with toxic levels for non-nervous tissue found by other investigators. Corssen and Skora (61) found 0.5 mM morphine had no effect and 1.3 mM morphine inhibited growth of "epithelial like" cervical carcinoma cells (not identified as a particular cell line). Notebloom and Mueller (50) found 0.1 mM morphine had no effect on the growth of HeLa monolayers and 1.0 mM morphine inhibited growth by 13%. Simon (49) found an LD_{50} of 0.5 mM for morphine on HeLa cells using a plating assay, with dextrorphan 6X and levorphanol 10X as potent. We find an RP_{50} (equivalent to LD_{50}) of 2.5 mM for morphine with dextrorphan 5X and levorphanol 7X as potent.

Suspension cultures were more sensitive to drugs than monolayer cultures (Table V). LM cells were inhibited 50% by 1.0 mM morphine. This could be due to an increased sensitivity of cells in suspension or due to the difference between LM and HeLa cells.

Tolerance Development

Plating Assay

An increase in tolerance was seen by cells grown by the protocol in Table III. An increase in tolerance as a function of days in cultivation in the presence of morphine is seen in Figure 4. A slight increase of tolerance is seen at 130 days. Tolerance is significant at 240 days, maximal at 415 days, with little change between 415 and 645 days, indicating a possible maximum in tolerance. Amphetamine, diphenhydramine•HCl, meprobamate, and morphine show significant increases in tolerance while caffeine, codeine, and propoxyphene show small increases in tolerance (Table VI). Although tolerance increases, the time course for development of tolerance is longer than that seen by other investigators. North and Martin (73) observed tolerance on the second addition of morphine to neuroblastoma cultures. Sasaki (55) showed tolerance to 166 μmolal morphine in 9 passages (18 days). Corssen and Skora (61) reported development of tolerance to 666 μmolal morphine in 9-15 passages. Ruffin *et al*. (62) did not observe an increase in tolerance during a 156 day experiment. The reason(s) for the differences in time required for tolerance development is unknown.

Morphological Tolerance

Morphological changes seen on addition of morphine include increase in vacuoles and formation of lipid droplets (55, 59, 61). Heubner *et al*. (59) used the decrease in these pathological symptoms as a measure of tolerance. We observe these changes on addition of morphine to control HeLa cells, but not on addition to tolerant cells.

Changes in colonial morphology are also seen during the training period. Colonies grown in the presence of morphine and phenobarbital revert to original colonial morphology upon removal of the drug, where those in the presence of acetylsalicylic acid, amphetamine, propoxyphene, meprobamate, diphenhydramine•HCl, or diazepam retain their modified morphologies.

Membrane Movement

Pomerat (140) observed continuous changes in the outline of plasma membranes and postulated a special importance for the mobility of the plasma membrane in interdigitation of cell boundaries. The plasma membranes of cells contain specific chemical receptors that regulate growth and metabolism of cells (81, 140, 184, 185) and the proposed opiate receptor (86-89). Corssen and Skora (61) described qualitative effects of morphine on cell movements. We also found morphine decreased cell movements using the assay described in Materials and Methods. The membrane movement of control cells is decreased by 50% using the RP_{50} morphine concentration from the plating assay. Tolerant cell membrane movement (0.08 μ^2/μ of length in the absence of drug) showed less inhibition in the presence of morphine than did control membrane (0.10 μ^2/μ of length in absence of morphine).

Cross Tolerance

Cross tolerance between various opiates is seen *in vivo* (2, 32) so that animals tolerant to morphine show tolerance to heroin. Heroin and morphine compete for the same receptor (87-89) and heroin is de-acylated to morphine, so the underlying tolerance mechanism(s) should be the

same. Saito (58) observed cross tolerance toward heroin and other opium alkaloids in cultures of iris epithelium addicted to morphine. We also find that HeLa cells tolerant to morphine (415 days) show the same relative tolerance to heroin as they do to morphine, indicating a possible common underlying mechanism(s) for tolerance, but the mechanism(s) is unknown.

Withdrawal

Sasaki (55) and Corssen and Skora (61) found that removal of morphine from tolerant cells caused a decrease in cell growth with subsequent cell death which could be reversed by addition of morphine. The requirement for morphine for growth is the equivalent of addiction and the decrease in the absence of drug is the equivalent of withdrawal. We find that cells trained with morphine, diphenhydramine•HCl, or amphetamine grow better in the presence of the appropriate drug than in its absence (Table IX) during a 12 or 13 day withdrawal experiment. Using cells withdrawn for 12 days (Table X) we find a better relative growth in the presence of low concentrations of drug for cells trained with diphenhydramine•HCl or morphine, with amphetamine showing a slightly lower relative growth. Using the relative plating assay we find a higher relative plating efficiency for cells in the presence of low concentrations of drug (up to 1.0 RP_{50}) for cells tolerant to amphetamine or diphenhydramine•HCl, but a decreased plating efficiency for morphine trained cells (Table XI). The differences in experimental conditions and disparity of responses observed with morphine suggest that different parameters contribute to the end measurement of growth rate in MDB and plating efficiency in petri dishes and that the

influence of cultivation in presence of drug or addiction on these parameters may be diverse enough to yield differentiation of responses.

Although the cells cultivated in the presence of morphine show tolerance using the plating assay, membrane movement, and withdrawal, it is not known whether these responses are due to a selection of cells with increased tolerance during cultivation or due to changes within the cell line.

Binding of [^{3}H]-Morphine

Morphine interacts with a specific morphine receptor (86-89) which is believed to exist on the plasma membrane. Our observations on the effect of morphine on plasma membranes may be interpreted as interaction with a receptor on the membrane. Binding of [^{3}H]-morphine was determined to ascertain if these cells have a specific morphine receptor. [^{3}H]-morphine rapidly binds to the cells, with maximal binding reached in 30 minutes, using the buffer system of Klee and Nirenberg (76). Klee and Nirenberg, however, found maximal binding in neuroblastoma cells was achieved in 10 minutes and remained constant for 60 minutes, and then decreased slightly. Figure 11 shows the effect of morphine concentration on binding. Binding of [^{3}H]-morphine is linear for both control and morphine tolerant HeLa cells. The amount of [^{3}H]-morphine bound to tolerant HeLa cells was higher than that bound to control cells, but saturation, which would be expected from a specific receptor (81) is not observed. Goldstein et al. (86) show that the morphine receptor not only shows saturation, but stereospecificity of binding, i.e., specific binding of [^{3}H]-levorphanol is decreased by exogenous levorphanol but not by the inactive D isomer,

dextrorphan. We used Pert and Snyder's (87) modification of Goldstein et al.'s assay (termination of incubation by filtration, collecting cells on GF/B filters) to ascertain the effects of levorphanol and dextrorphan on [^{3}H]-morphine binding (Table XII). Both dextrorphan and levorphanol inhibited binding of [^{3}H]-morphine by about the same amount in tolerant and control HeLa cells. In Table XIII shows the displacement of [^{3}H]-morphine binding by various drugs. All of the drugs displaced [^{3}H]-morphine with 10-50% displacement in the first minute. There is no specific pattern to the displacement by the various drugs. The displacement of [^{3}H]-morphine by non-opiates as well as opiates indicate non-specific binding, and since the displacement is rapid, it indicates a "loose" binding. While there is no evidence that binding is stereospecific, there is a significant difference in the amount of [^{3}H]-morphine bound to tolerant and control cells (Figure 11, Table XII). This change might be responsible for the cells' ability to grow in medium containing morphine.

Relationships between specific receptors and biological effects is unclear. The opiate receptors have only been shown in nervous tissue (186), neuroblastoma cultures (76), mouse vas deferens (187), and guinea pig illium myenteric plexus (97), but tolerance and addiction occurs in non-nervous tissue (52-61). Furthermore, no changes in number, binding affinity, or stereospecificity (100) of receptors occurs during development of tolerance or addiction, and receptor concentration varies from 2.5×10^4 (101) to 1.5×10^{10} (103) receptors/cell with little difference in analgesic response.

The morphine trained HeLa cells used in these binding experiments are cells which were frozen after 645 days of cultivation in the

presence of morphine, stored frozen for 1 year, re-initiated, and maintained for 450 days at 1.0 RP_{50} morphine. This was necessitated after a combination of a severe storm (water in tissue culture lab) and loss of air conditioning led to mold contamination of the laboratory. All ongoing stocks (all contaminated) had to be terminated, with the lab totally stripped, cleaned, and re-sterilized (~8 months work). Unfortunately, the re-initiated cells no longer show the increased tolerance with the plating assay when compared to stock HeLa that was seen previously. However, these cells were able to grow well in 1.0 RP_{50} morphine whereas the control HeLa cells die within three passages. Since the re-initiated line shows different properties, it is unknown as to whether the cells which were used for tolerance measurements possessed a receptor.

Glass Fiber Filters

In addition to the measurement of toxicity, we wished to determine the effect of morphine on biochemical processes, specifically nucleic acid synthesis and protein synthesis. The incorporation of [^{3}H]-thymidine can be used to measure DNA synthesis, the incorporation of [^{3}H]-uridine can be used to measure RNA synthesis, and incorporation of [^{3}H]-tyrosine to measure protein synthesis (156). The difference between the uptake and incorporation is the amount transported into the intracellular pool (157).

Several methods exist for growth of cells to measure the effect of parameters on uptake and incorporation of various precursors. Suspension cultures are the method of choice since they can be readily labelled and easily sampled at various time points. HeLa cells,

however, do not adapt readily, so methods for labelling monolayer cultures must be used. To avoid loss on transfer, it would be preferable to grow the cells on the supporting material. This has been previously reported for plastic discs (153), glass coverslips (152), membrane filters (188), and glass scintillation vials (154). These supports are not easy to wash, so we developed an assay using glass fiber filters as growth supports. Table XIV shows a comparison of uptake of [^{3}H]-thymidine by HeLa cells grown on various supports. The two glass fiber filters used as supports showed the highest uptake. Whatman GF/A filters were chosen for further determinations. Cells grown on GF/A filters show essentially identical uptake to those grown in MDB (slope of log CPM vs log cells/support for GF/A is 0.838 ± 0.002 and for MDB is 0.842 ± 0.004). Cleland's (183) application of the student's t test on the slopes showed no significant difference (t 0.2, 46). There is a slight deviation from linearity by cells grown on GF/A filters at 10^6 cells/support due to saturation of the filter with cells. Both supports gave the same results, but with the GF/A cultures the triplicates are in the same petri dish and require less medium (10 ml versus 30 for MDB), less labelled precursor (5.0 µCi versus 15.0 µCi for MDB), and less manipulation (filters only have to be removed and rinsed under aspiration, MDB have to be scraped, cells collected on a filter, and filter then rinsed) than do the MDB cultures. In addition, background was lower (70 CPM versus 850 CPM for MDB).

The applicability of the method for measuring synthesis of macromolecules is shown for DNA in Figure 13, for RNA in Figure 14, and for protein in Figure 15. In all cases, uptakes and incorporations are

linear with respect to number of cells/filter, concentration of labelled precursor, and duration of pulse. Higher error is seen with [^{3}H]-tyrosine as a precursor due to its low specific activity (0.43 Ci/mM) and the high tyrosine content of medium 199. The validity of the method for other cell lines is shown by the uptake and incorporation of [^{3}H]-TdR by LM cells (Figure 16).

The effect of hydroxyurea (an inhibitor of DNA synthesis) on uptake and incorporation of [^{3}H]-TdR is shown in Figures 17 and 18. Morphine did not affect the relative uptake or incorporation of [^{3}H]-TdR as can be seen in Table XV. The lack of effect of morphine combined with the lack of response of trained cells precluded further testing.

The use of glass fiber filters as supports offers several advantages. The glass fiber filters are less expensive than millipore filters (1/3 the price) and show superior [^{3}H]-TdR incorporation (Table XIV). Other advantages include: 1) replicates are incubated in the same petri dish under identical conditions (up to 6 filters can be handled per petri dish using a modified petri dish); 2) less medium, less radioactive precursors, and fewer cells are required; rapid termination of the reaction, easy washing, and selective treatment of the precipitate can be done by filtration; 4) high counting efficiency is obtained by [^{3}H]-labelled compounds on glass filters (189) (we also observed no quenching of [^{3}H]-TdR in the presence of GF/A filters, but found 10% quenching by millipore filters); and 5) the filters are made of borosilicate glass which is preferable for the growth of delicate cells (190).

CHAPTER VI

SUMMARY

The toxicities of various drugs to cells in culture were measured using a plating assay for HeLa cells and a growth assay for LM cells (suspension). The compounds were more toxic to LM cells than to HeLa cells, but whether this is due to the difference in cell lines or difference in susceptibility of cells grown in suspension versus cells grown on a surface was not determined. The RP_{50} of the opiates tested (codeine, 1.0 mM, dextrorphan, 0.51 mM, heroin, 1.3 mM, levorphanol, 0.37 mM, leucine enkephalin, 0.13 mM, morphine, 2.5 mM and nalline (antagonist), 0.0275 mM) are in general agreement with toxicity measurements on non-neuronal cells made by other investigators. Due to the nature of the plating assay, toxicity could be due to one or two factors, i.e. decrease in growth rate or decrease in attachment of cells to substratum. The opiates tested (codeine and morphine) did not affect attachment, but several other drugs tested did.

Lines of HeLa cells which show increased tolerance (measured by the plating assay) to various drugs (amphetamine, caffeine, codeine, diphenhydramine•HCl, meprobamate, morphine, and propoxyphene) were developed. All cell lines were frozen at the 645 day level. Only the morphine tolerant line was maintained after this point for further testing. The time required for development of tolerance in HeLa cultures was longer than observed in vivo or with other cell lines by

other investigators. However, there is marked variation in the times others found necessary. Maximum tolerance (as measured by the plating assay) was achieved for most drugs at 415 days with little change between 415-645 days.

Membrane movement of tolerant and control HeLa cells was measured by filming the cells using time lapse cinematography and tracing membrane projection as a function of time. HeLa cells show inhibition of membrane movement by morphine, with 1.0 RP_{50} morphine decreasing movement by 50%. Tolerant HeLa cells show less inhibition of membrane movement in the presence of morphine than do control HeLa cells.

Cells tolerant to amphetamine, diphenhydramine•HCl, and morphine show slightly decreased growth in the absence of drug compared to growth in its presence (addiction), but do not show complete loss of viability seen by early workers.

Cells cultivated in the presence of morphine are cross tolerant to heroin. Cellular basis for tolerance was not determined.

Laboratory contamination caused termination of the continually maintained tolerant morphine line. The frozen morphine tolerant HeLa line (645 days) was re-initiated after decontamination of the laboratory (~8 months work). This line no longer showed increased tolerance as measured by the plating assay, but did grow well when maintained at 1.0 RP_{50} morphine, while control HeLa's could not. This line was terminated at the end of the morphine binding studies.

Since the properties of the cells used for the binding assays are different than those used for the plating assays which demonstrate tolerance development, one can not categorically conclude that the original cells lack specific morphine receptors.

Binding of [^{3}H]-morphine to HeLa cells was studied to determine if HeLa cells (tolerant or control) possess an opiate receptor. Binding of [^{3}H]-morphine was linear with morphine concentration, with no evidence of saturation or stereospecificity. There was ready displacement by a variety of drugs. No evidence for an opiate receptor was seen in control cells or cells cultivated in the presence of morphine. Binding of [^{3}H]-morphine to tolerant HeLa cells was consistently higher than to control HeLa cells.

The utilization of glass fiber filters as a substrate for cell growth and for subsequent utilization for biosynthetic analysis was determined. Uptake and incorporation of [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-tyrosine was linear with respect to cells plated/filter, concentration of label, and duration of pulse, showing applicability to measurement of DNA, RNA, and protein biosynthesis. The uptake of [^{3}H]-TdR by HeLa cells on GF/A filters was superior to that of other supports tested. Uptake of [^{3}H]-TdR by LM cells grown on GF/A filters is also linear with respect to cell number, showing the method is applicable to other cell types. The utilization of cells grown on GF/A filters to measure inhibition of uptake and incorporation was studied. Hydroxyurea, a known inhibitor of thymidine uptake and incorporation inhibited uptake and incorporation of [^{3}H]-TdR by HeLa cells grown on GF/A filters. Morphine had no effect on uptake or incorporation of [^{3}H]-TdR by HeLa cells grown on GF/A filters.

The utilization of GF/A filters as a growth substrate in these studies has several advantages, including: higher uptake, lower cost than membrane filters, less medium and less precursor required, ease of handling, no loss of cells in transfer, selective treatment of

precipitate and ease of termination of pulse (rinsing filters under vacuum aspiration), growth of cells on borosilicate glass (preferred), and replicate cultures (3-6 filters) can be handled on the same petri dish under the same conditions. Using a 2 hr attachment period and 4 hr pulse, the experiments can be completed in a single day.

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