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Observations on the effects of aspirin on cells in vitro

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OBSERVATIONS ON THE EFFECTS OF ASPIRIN ON CELLS IN VITRO

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by

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INTRODUCTION

INTRODUCTION

Numerous reports concerned with the effects of chemical agents (drugs) on animals, especially humans, have appeared in the literature over a period of centuries. Until very recently, however, the observations on which these reports were based have been rather general. The salicylates, for example, have been used and studied for many years yet investigations into their specific effects within living cells are minimal.

The term salicylates is used to denote a group of agents (relatively simple in chemical structure) that have the 2-hydroxybenzoate radical in common, many members of which have been used as medicaments since ancient times. Preparations from the barks, leaves and fruits of plants containing salicylates and glycosides of salicyl alcohol were among the earliest pharmacist's inventories. The first synthetic salicylates appeared in the latter part of the nineteenth century.

Of the several members of the salicylate group, the two that have gained widespread use in clinical practice are sodium salicylate and acetylsalicylic acid, commonly known as aspirin, the name of which was derived from the contraction of the word acetyl, plus the word spirin, from Spiraea, a plant whose members contain salicylate compounds.

Although investigations on the in vivo effects of the salicylates began many years ago, little is known about the basic nature of their effects.

Today, the salicylates are used mainly as antipryetics, analgesics, and particularly as anti-inflammatories in rheumatic disorders.

For analgesia, it has been found that the plasma level of salicylate must be about 16.1 mg%, while acetylsalicylic acid is effective in concentrations as low as 3.1 mg%. For this reason, acetylsalicylic acid is often preferred to other salicylates. Its effectiveness at this lower concentration may be related to the fact that it is a weaker acid, is less ionized at low pH, and is therefore more lipid soluble, allowing it easier access to the pain receptors. It is not known, however, if actual access is necessary. Salicylates' effect may be due, instead, to blocking or occupying the surface pores of the chemoreceptors for pain. There is also the possibility that salicylate analgesia may be the result of inhibition of electron transport and the coupling mechanism which accomplishes oxidative phosphorylation, as suggested by Brody (1956).

According to Smith and Smith (1966), in rheumatic fever therapy plasma salicylate levels reach about 20 to 30 mg%; toxic symptoms in vivo appear as the level is increased to approximately 35 mg%. These symptoms are related to alterations in the acid-base balance in the blood, disturbances in carbohydrate metabolism, and certain factors that result in gastrointestinal hemorrhage. Salicylates can be lethal in humans at plasma levels as low as 50 mg%. The actual dose depends largely upon the age and condition of the subject.

In order to understand more fully the mode of action of the salicylates, attempts must be made to pinpoint the sites of their action. Although a considerable amount of work has been done on the effects of salicylates on the whole animal, relatively few investigations have been made at the cell, tissue and organ levels. However, in recent years, investigations at these levels have increased

so that suggestions as to the manner in which salicylates exert their influence are becoming more meaningful.

Several early investigators including Singer (1901), working with rabbits, Barbour and Devenis (1919), and Cochran (1954) (with humans) have shown that the salicylates cause an increase in oxygen consumption in whole animals. Smith and Jeffrey (1956), using isolated rat diaphragm, Brody (1956), with mouse liver and muscle tissue, Sproull (1957), with rat brain and mouse liver slices, and Penniall (1958), with rat liver mitochondria, have shown a similar increase in oxygen consumption in vitro. This action seems to be linked to the interference by salicylates of two important groups of enzymes, the hydrogenases and those related to oxidative phosphorylation.

That salicylates at low concentrations are capable of uncoupling oxidative phosphorylation was first suggested by Brody (1956), and Brody (1956) and Jeffrey and Smith (1959) discovered that phosphorylations along the whole respiratory chain seem to be affected. However, Penniall (1958) has shown that certain oxidative phosphorylations may be more susceptible to the effect of salicylates than others, as in the case of the terminal phosphorylation (cytochrome to oxygen). The direct effects appear to be only on the phosphorylations and not on the oxidative mechanism.

The mechanism by which uncoupling takes place is not known. Charnock and Opit (1962) believe that it may be related to an effect on the permeability of the mitochondrial membrane. They have demonstrated increased permeability to ATP and a loss of potassium ions in mitochondria exposed to salicylate. Another

possible mechanism is the direct inhibition of one or more of the enzymes involved.

Lutwak-Mann (1942), using rat liver and kidney slices, Fishgold, Field, and Hall (1951), using rat brain and liver, and Sproull (1954), with rat brain slices, have shown that high concentrations of salicylate decrease oxygen consumption *in vitro*. This effect seems to be due to their action on at least two steps in the tricarboxylic acid cycle (involving alpha-keto-glutarate dehydrogenase and succinate dehydrogenase). Investigations by Bryant, Smith, and Hines (1963) and Hines and Smith (1964), using rat liver mitochondria, indicate that the action of several dehydrogenases are inhibited *in vitro*. This inhibition is thought to be due to a reversible competition with pyridine nucleotide coenzymes. According to Smith and Smith (1966), enzymes requiring flavin nucleotides may also be affected. Recent studies by Dawkins, Gould, Sturman, and Smith (1967) tend to confirm these observations.

The salicylates have been shown to affect carbohydrate metabolism and some *in vivo* studies have been concerned more specifically with their effect on blood sugar levels; however, the results of this work are somewhat contradictory. This action seems to be related to their effect on oxidative phosphorylation, to an increase in the uptake of glucose, and to an increase in the rate of tissue glycolysis. A decrease in the rate of synthesis of glucose from non-carbohydrate precursors and an increase in the rate of glycogenolysis have also been suggested by Smith and Smith (1966).

Salicylates seem to affect fat metabolism but, here too, reports are conflicting. Niederland (1963a, 1963b) has shown that doses of 230 to 690 mg./Kg.

of body weight caused an increase in neutral fat deposition in the liver and kidney of rabbits. This seems to indicate that either lipogenesis is increased or there is interference in fat removal or metabolism within the organs (eg. the uncoupling action of salicylates may inhibit fatty acid oxidation). Both of the preceding possibilities may be working together.

Carlson and Ostman (1961) have reported that the administration of 5 grams of aspirin to normal and diabetic humans has caused a decrease in free fatty acids within the plasma. Using labelled palmitate, they found that its rate of removal from the circulatory system was not altered, and concluded that aspirin depresses the release of fatty acids from adipose tissue. In vitro experiments using rat epididymal fat bodies in the presence of salicylate have shown a decrease in the release of free fatty acids.

Reports on the effects of salicylates on protein and amino acid metabolism are likewise conflicting. Manchester et al (1958), using isolated rat diaphragm, and Bellamy et al (1963), using rat costal cartilage, have indicated that salicylates inhibit the in vitro incorporation of amino acids into protein while Weiss et al (1962) suggested increased incorporation. Whatever the case may be, their effect could involve an uncoupling action (which may cause increases in protein catabolism).

Huggins et al (1961), employing rat liver, brain, and heart muscle tissues, have suggested that salicylates cause an increase in the incorporation of pyruvate into glutamic acid. Further investigations indicated that practically every enzyme system using glutamate as a substrate was inhibited by the action of

salicylate in vitro, but the mechanisms appear to differ in the various systems.

Salicylates are known to inhibit the following glutamate pathways in vitro: (1) Conversion to glutamine, (2) decarboxylation to gamma-amino-butyrate, (3) incorporation into proteins, (4) aminotransferase reactions involving glutamate, (5) conversion to proline, and (6) oxidative deamination to alpha-oxoglutarate.

Bergel and Bray (1956, 1959) have reported that salicylates inhibit xanthine oxidase activity in cow's milk. This appears to be related to their effect on the flavin nucleotide enzyme involved. Mitidieri and Affonso (1959) have reported similar action in rats.

Bellamy, Huggins, and Smith (1963) have indicated that salicylates affect the biogenesis of some forms of collagen and collagen-like proteins; Bostrom et al (1963), using bovine heart valves, and Whitehouse et al (1961, 1962, 1963, 1964), using bovine cartilage and cornea and rat cartilage, have demonstrated an inhibition of the synthesis of mucopolysaccharide sulfates in vitro. Bostrom et al (1964) have also shown an in vivo inhibition in rats. These effects are possibly related to those observed on oxidative phosphorylation and on the amino acid transferase enzymes.

Much consideration has been given to the role of uncoupling agents in anti-inflammatory action; however, Thompkins and Lee (1969) have recently reported that acetylsalicylic acid does not cause the uncoupling of oxidative phosphorylation. Using mitochondrial preparations they have shown that the uncoupling

action previously reported is due to one of the hydrolytic products, salicylic acid. They have also pointed out that one compound that is known to be a strong uncoupler, 2,4 dinitrophenol, has neither anti-inflammatory nor anti-rheumatic action; however, one must also consider the in vivo situation; 2,4 dinitrophenol is rapidly metabolized in the liver and very little of the intact agent ever reaches the site of inflammation. Nevertheless, Winder et al (1958) and Stenger (1959) have indicated that somewhat heavy to lethal doses of this compound do show moderate activity. Waltner et al (1959) do not agree with these observations. Since aspirin uncouples only as the result of the formation of salicylic acid, and is a more potent anti-inflammatory agent than salicylate alone, it is obvious that further investigations in this area are necessary.

Because of the relatively few reports appearing in the literature on the effects of salicylates at the cellular level and because of the rather controversial results obtained, the following investigations were made.

MATERIALS AND METHODS

MATERIALS AND METHODS

Origin and Nature of the Cells Used

Three different types of cells were used for this study. The first type, designated as NCTC clone 2555 (low tumor-producing fibroblasts), was originally derived from a culture of subcutaneous areolar and adipose connective tissue of a normal 82 day-old C3H/HeN mouse by Likely, Sanford, and Earle (1952). Sanford, Likely and Earle (1954) found that cultures of this parent strain produced sarcomas in about one percent of mice injected, but, under similar conditions, clone 2555 produced no tumors. It has been cultured since its origin in antibiotic-free NCTC 109 plus 10% newborn calf serum.

The second type of cell, designated as PtK_1, was derived from the kidney of an adult female rat kangaroo, Potorous tridactylis (a marsupial). It is referred to as the K strain. This line was isolated and carried in Eagle's minimum essential medium (MEM) with non-essential amino acids and Earle's BSS with reduced bicarbonate (0.85 gm./liter) plus 10% newborn calf serum and antibiotics. Stock cells are maintained in NCTC 109 plus 10% newborn calf serum and antibiotics. Experimental cells were cultured in the same medium without antibiotics.

The third type of cells used in these experiments was derived from the diploid PtK_1 which transformed in our laboratory in January of 1969 and are now polyploid. Since then they have been referred to as Kc cells and their rapid rate of proliferation makes them extremely useful in preliminary studies.

Cultivation of Cells

All cells were cultivated in pyrex T-flasks with silicone stoppers. Stock cultures were carried in T-60's (bottom surface approximately 60 cm^2) and experimental cultures in T-15's (approximately 15 cm^2). The fairly suitable optical properties of these T-flasks make their use desirable if microscopic examination of the cultures is necessary.

Before use, all T-flasks were immersed in dichromic acid for a period of not less than twelve hours, and then rinsed in tap water fifteen times and in distilled water three times. Other glassware and stoppers were soaked in detergent for twelve hours and rinsed in tap water fifteen times and in distilled water three times. After washing, all were dried and sterilized for 20 minutes (250°F. at 20 lbs.).

The cells were cultured in NCTC 109 plus 10% newborn calf serum (GBI Powdered Medium, NCTC 109 - General Biochemicals, Chagrin Falls, Ohio). The serum was added at the time of medium preparation. The prepared medium is filtered through a series of prefilters and finally through a millipore filter with a pore size of 0.22 micron. It is then stored in 500 ml. serum bottles at 4° C. until use, at which time it is warmed to approximately room temperature and again filtered through a millipore filter with a pore size of 0.22 micron.

Replication Technique

All cultures other than stocks were set up in the replicate method, as modified by Pace and Aftonomos (1957). The stock cultures were maintained in

T-60's. In the case of Low Line and transformed K's, the cells were removed by scraping with a nichrome scraper. The diploid K's were removed with the aid of 0.5% trypsin. After removal the suspended cells were centrifuged at 750 rpm for ten minutes. The trypsin was then decanted and medium added. If necessary, the cell clumps were broken up by pipetting. They were then placed in the replicator and diluted to the desired number with medium.

Replicate cultures were set up adding 2 ml. of the cell suspension to each T-15. Each flask was then gassed with a mixture of 95% air and 5% CO_2, sealed with a silicone stopper, and placed in an incubator at 37° C.

Growth Measurement

In this study, increase in cell number was taken as an indication of growth. Cell counts were made with the aid of a Coulter electronic counter (model B), which operates on the principal of "electronic gating". To operate this instrument, the cells to be counted were first placed in a special counting solution which causes the cells to separate and has the necessary electrolytic properties for the counter. Once the cells had been separated, they were suitably diluted (normally to 25 ml.) and then suspended equally by pouring from one vessel to another several times.

Counting was accomplished as the cells were drawn into a small aperture (100 microns) which tends to prevent the passage of several cells at one time. As the cells passed through, they also were drawn between two platinum electrodes across which a current was flowing. This current was reduced by the cell's

passage due to its poor conductivity (electrolytic displacement). The reduction in current was recorded and read out by a decade counting system. The amount of sample drawn in was governed by a mercury manometer which automatically starts the count and stops it after 0.5 ml. of the sample has been monitored, which takes approximately 15 seconds.

Coincidence losses were corrected by a coincidence correction factor of plus 3%. For counts over ten thousand on the counter, a coincidence correction chart supplied by the manufacturer was used. Error is from one to two percent.

In all cases, counts were made on at least two cultures (often three).

Experimental Procedure

Following replication, the cells were allowed 48 hours to attach and adjust before being subjected to experimental conditions.

Aspirin was dissolved in the medium by stirring with the aid of a magnetic stirring device. Once the highest concentration to be used was prepared, it was diluted with the amount of medium necessary to give the lower concentrations desired.

When replacing medium in the replicate cultures, that of the control cultures was first drawn off, using an aspirator and Pasteur pipettes. As each culture was evacuated, fresh medium was added by means of a duplicator which is capable of delivering a pre-set amount (in this case 2 ml.) with accuracy. Each flask was then gassed with a mixture of 95% air and 5% CO_2. Once the medium of the control cultures was changed, each of the successively higher concentration

groups was changed. The duplicator was flushed with each higher concentration before that concentration was used.

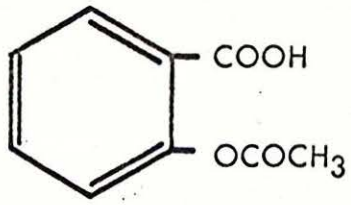
The same procedure was followed for cultures in Leighton tubes, set up for histochemical studies.

Cells used for time lapse studies were placed in Rose chambers. The medium to be used was first gassed with 95% air and 5% CO_2 and then sterilized by filtration. Cells were injected into the chamber and the medium changed by means of affixed hypodermic needles. When not being used they were sealed with parafilm.

Motion pictures were made with the aid of a Sage cinematographic apparatus and a Bolex 16 mm. camera. The microscope was an inverted Model M Nikon. All studies were done in phase contrast.

Nature of the Chemical Agent

Acetylsalicylic acid exists mainly in the form of elongated monoclinic crystals. It is colorless and odorless with a solubility of 250 mg. per ml. at 20° C. Its chemical formula is $CH_3COOC_6H_4COOH$ with a molecular weight of 180.15:



Although aspirin is stable under normal circumstances, exposure to moist air results in gradual hydrolysis to acetic acid and salicylic acid. For this reason, care must be taken to keep the compound dry by tightly securing the

cap and placing it in a dessicator when not in use.

Because aspirin is not stable in solution, fresh mixtures were made before each use.

Chemical Analysis of the Media

Forty-eight hour samples of media were analyzed for glucose and lactate by colorimetric methods.

Glucose was assayed by Hultman's method as modified by Hyvarinen and Nikkili (1962) which is relatively free of interference, gives highly reproducable results, and has good sensitivity and specificity while retaining the simplicity of the original method.

Lactate was determined by the method of Scholz et. al. (1959). In this method lactate dehydrogenase catalyzes the reaction:

$$\text{1-lactate} + \text{DPN} \xrightleftharpoons{\text{LDH}} \text{Pyruvate} + \text{DPNH}^{+} + \text{H}^{+}.$$

Although equilibrium favors the formation of lactate and DPN, it can be shifted completely in favor of pyruvate and DPNH+ by using hydrazine to trap the formed pyruvate, an alkaline medium, and an excess of DPN. The amount of DPNH+ produced was ascertained by means of a Coleman Hitachi 101 Spectrophotometer (340 mμ).

Cells for cytochemical determinations were cultured on slides or coverslips in Leighton tubes.

Low Line cells exposed to various concentrations of aspirin were assayed for fats and cytochrome oxidase activity. For fats, Oil-Red-O stain was used;

it imparts a pink to red color to the fat droplets. Cytochrome oxidase activity was determined by the method of Burstone (1961).

Succinic dehydrogenase activity in K strain cells was ascertained by the Method of Seligman and Rutenburg (1951).

RESULTS

RESULTS

The Toxic Level of Aspirin

For the determination of the toxic level of aspirin both Low Line and K cells were used. The cells were cultured in T-15 flasks and exposed to various concentrations of the drug. Preliminary work involved visual observation only, while later studies involved both visual observation and the study of growth in terms of cell number.

Using Low Line cells it was found that a concentration of 200 mg.% caused toxic symptoms to appear within 24 hours. Lower concentrations (down to 60 mg.%) caused rounding of cells, increased vacuolization, appearance of unidentified granules, and an increase in number of "giant" cells within approximately 72 hours.

Two consecutive runs were then made using concentrations of 50, 40, 30, and 10 mg.%. The cells were counted every two days and a graph of the growth in one experiment is presented in Figure 1A. There was an increased proliferation in the cultures in which the cells were exposed to 10 mg.% aspirin, while the higher concentrations had an increasingly greater inhibitory effect.

In both runs, toxic effects in 50 mg.% appeared within 24 hours after exposure. By 72 hours, many of these cells, as well as those exposed to concentrations of 40 and 30 mg.%, had large vacuoles and granules, and appeared to become less opaque. There was also an increase in the number of "giant" cells.

Within eight days, the cells in 50 mg.% and 40 mg.% took on a spherical shape and relatively few remained attached. Some showed large

vacuoles and some showed a small number of large granules. In 30 mg.% effects were noticeable and, although there were an unusual number of dead cells in the medium, growth was fairly heavy.

During the ten days of observation, the cells in 10 mg.% appeared to be similar, morphologically, to those of the controls; however, proliferation was greater than in the control cultures and more vacuoles or oil droplets appeared by the eighth day. The effects of concentrations of 10 mg.% and 50 mg.% at five days are shown in Figure 2.

In order to ascertain whether or not there were any differences in toxicity or other effects in different cell species, Rat Kangaroo cells were exposed to 100, 75, 50, and 25 mg.% aspirin. The results are presented in Figure 3.

Within 72 hours, the cells subjected to 100 mg.% showed an increase in number of vacuoles; the outlines of the cells were much more distinct than those of the controls; some of them appeared to have undergone "shrinking" and many were floating in the medium.

When exposed to 75 mg.%, the cells demonstrated similar effects but they were less obvious. In 50 mg.% they exhibited even fewer, and in 25 mg.%, they showed little, if any, effects.

Within six days, most of the cells in 100 mg.% had become spherical and many of them were dead. In 75 mg.%, some toxic effects were evident, but in 50 mg.% and 25 mg.%, no obvious effects were evident.

By eight days, most, if not all, of the cells in 100 mg.% aspirin were dead. In the 75 mg.%, there were many dead cells present, but there were also

FIGURE 1

The effects of aspirin on Low Line cells.

A. Growth

B. The amount of glucose utilized per cell

C. The amount of lactate produced per cell

FIGURE 1

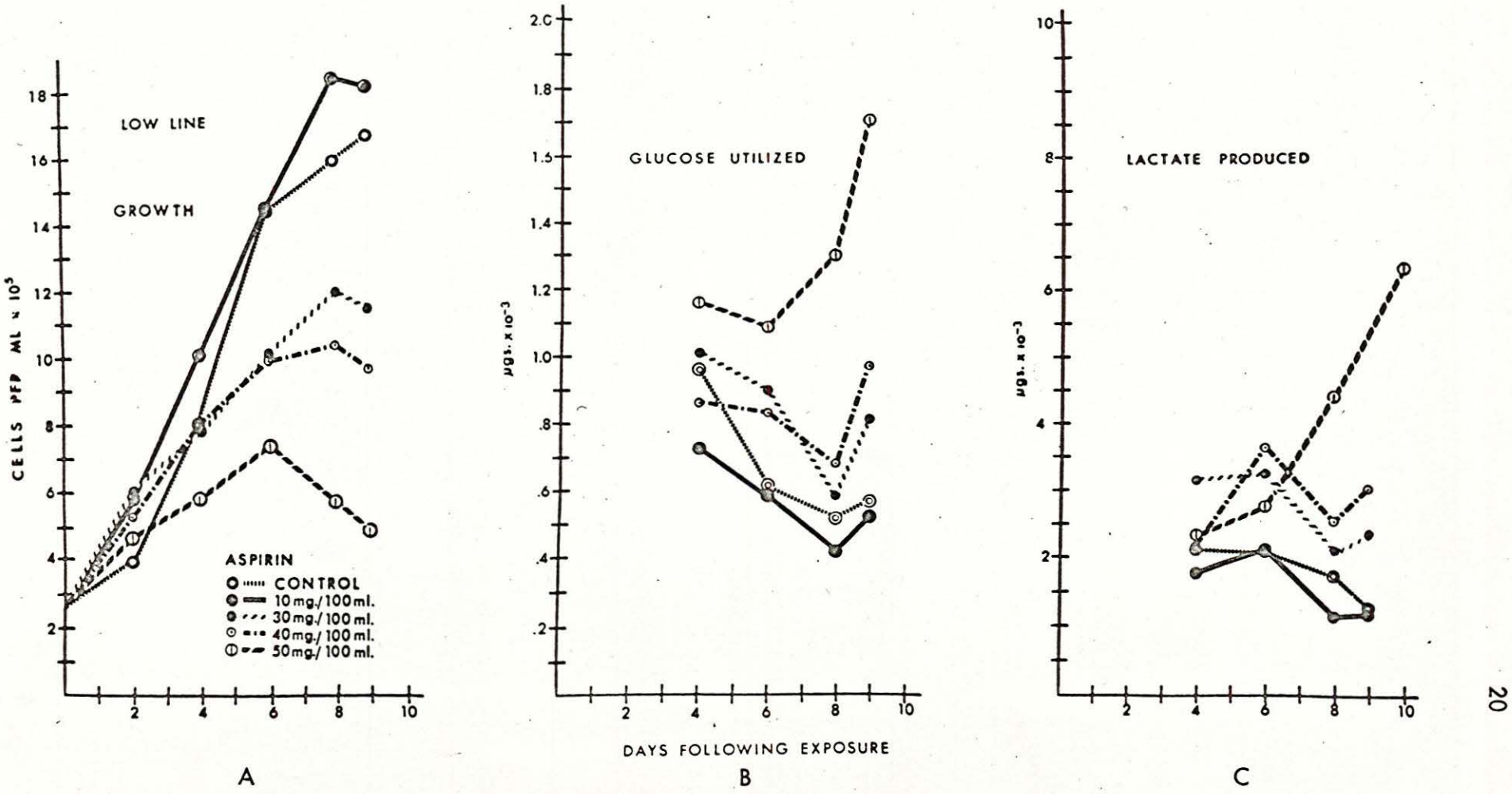

LOW LINE
GROWTH
CELLS PFD ML x 10³
ASPIRIN
CONTROL
10 mg./100 ml.
30 mg./100 ml.
40 mg./100 ml.
50 mg./100 ml.
A
GLUCOSE UTILIZED
µgs. x 10⁻³
DAYS FOLLOWING EXPOSURE
B
LACTATE PRODUCED
µgs. x 10⁻³
C

FIGURE 2

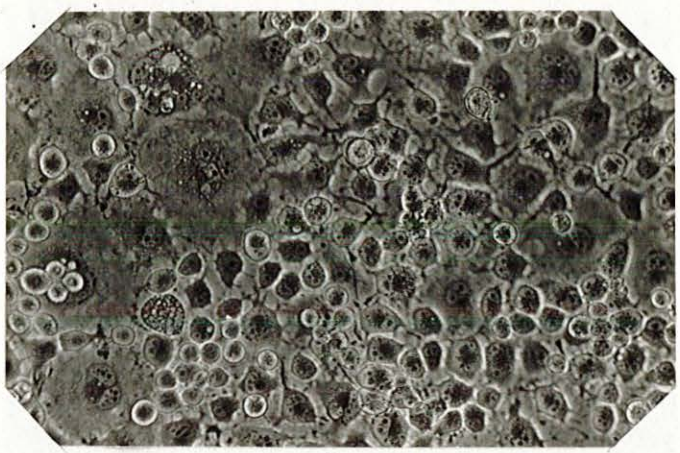
The effects of aspirin on Low Line cells exposed for a period of five days.

A. Control

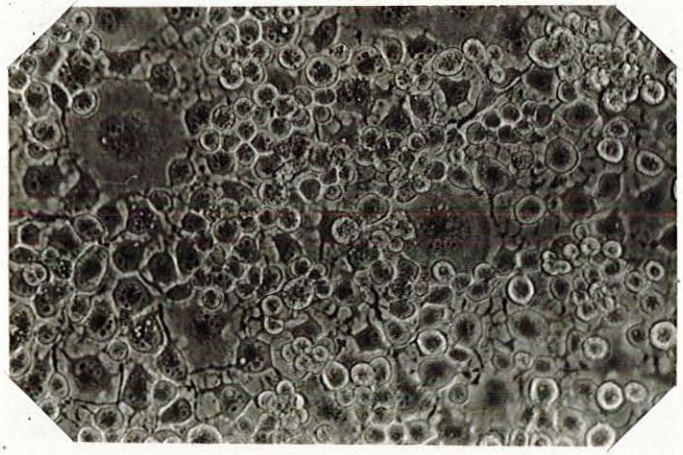
B. A concentration of 10 mg.% has resulted in an increase in cell density; no other effects are seen.

C. A concentration of 50 mg.% has resulted in a significant inhibition of growth as indicated by the relatively low cell density. Increased vacuolization and granulation are also obvious.

FIGURE 2



A

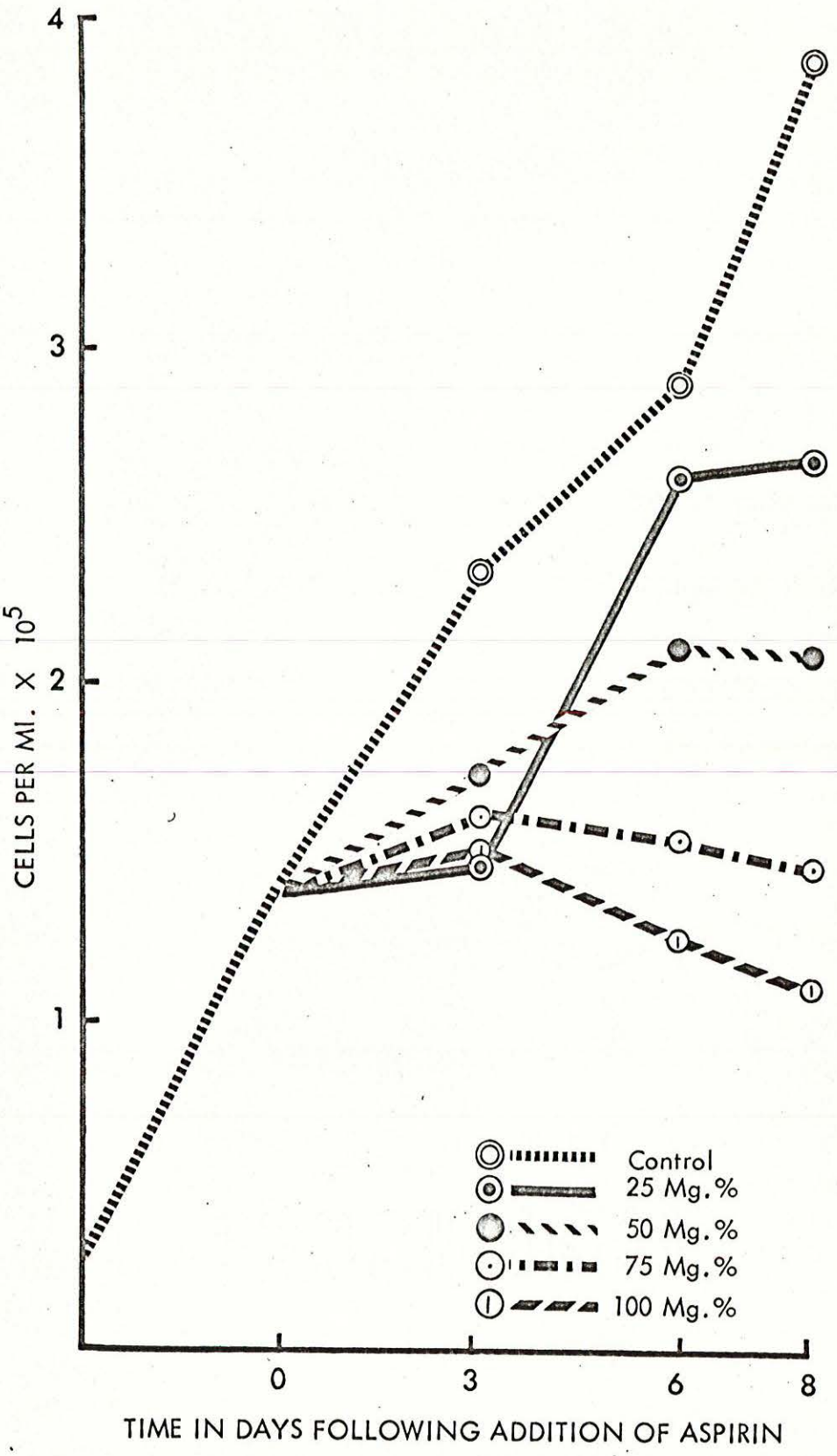


B

C

FIGURE 3. The effects of various concentrations of aspirin upon the growth of K cells.

FIGURE 3


4
3
2
1
CELLS PER Ml. X 10^5
0
3
6
8
TIME IN DAYS FOLLOWING ADDITION OF ASPIRIN
Control
25 Mg.%
50 Mg.%
75 Mg.%
100 Mg.%

many viable cells. In the 50 mg.%, there were some toxic symptoms evident, but growth was still heavy. The cells in 25 mg.% were similar in appearance to the controls.

It is interesting to note that the toxic level *in vitro* is in the same general range as the toxic plasma level *in vivo*.

Transformed K cells were also studied (for growth effects only). Aspirin concentrations of 50 mg. and 10 mg.% were used. The results are shown in Figure 4. Although little, if any, difference between control cells and cells exposed to 10 mg.% was evident, 50 mg.% appeared to be distinctly inhibitory to growth.

Glucose Utilization and Lactate Production

Glucose utilization and lactate production were also ascertained for Low Line cells when exposed to various concentrations of aspirin. The results are presented graphically in Figures 1B and C. From these results it is apparent that, with the exception of 10 mg.%, increases in aspirin concentrations resulted in corresponding increases in both glucose utilization and lactate production. The 10 mg.% concentration resulted in a decrease in both of these.

Table I shows the compiled results of the effect of aspirin on growth, glucose utilization, and lactate production in Low Line cells; in addition, the ratio of lactate produced to glucose utilized is also presented. These ratios are displayed in Figure 5. With the exception of the cells exposed to 50 mg.%, all experimental cells showed a higher glycolytic ratio than control cells at four and six days of exposure. By nine days, ratios for cells exposed to the various

FIGURE 4. The effects of various concentrations of aspirin upon the growth of transformed K cells.

FIGURE 4

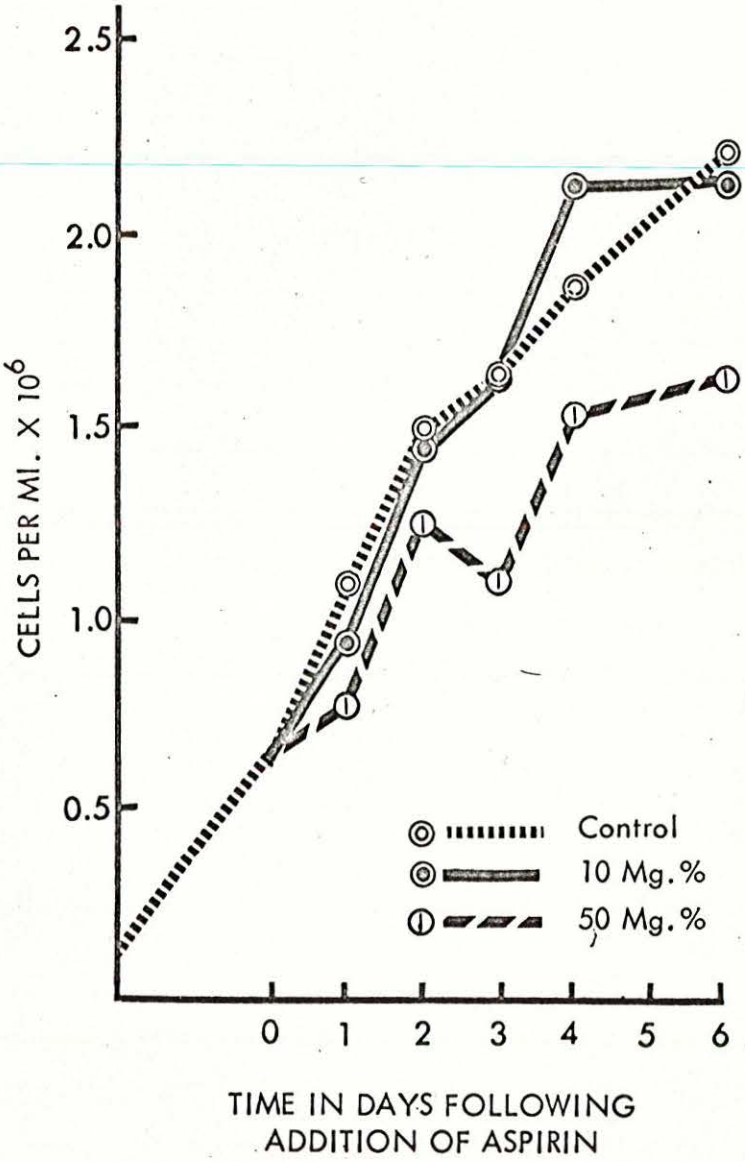

2.5
2.0
1.5
1.0
0.5
CELLS PER ML. X 10^6
0
1
2
3
4
5
6
Control
10 Mg.%
50 Mg.%
TIME IN DAYS FOLLOWING
ADDITION OF ASPIRIN

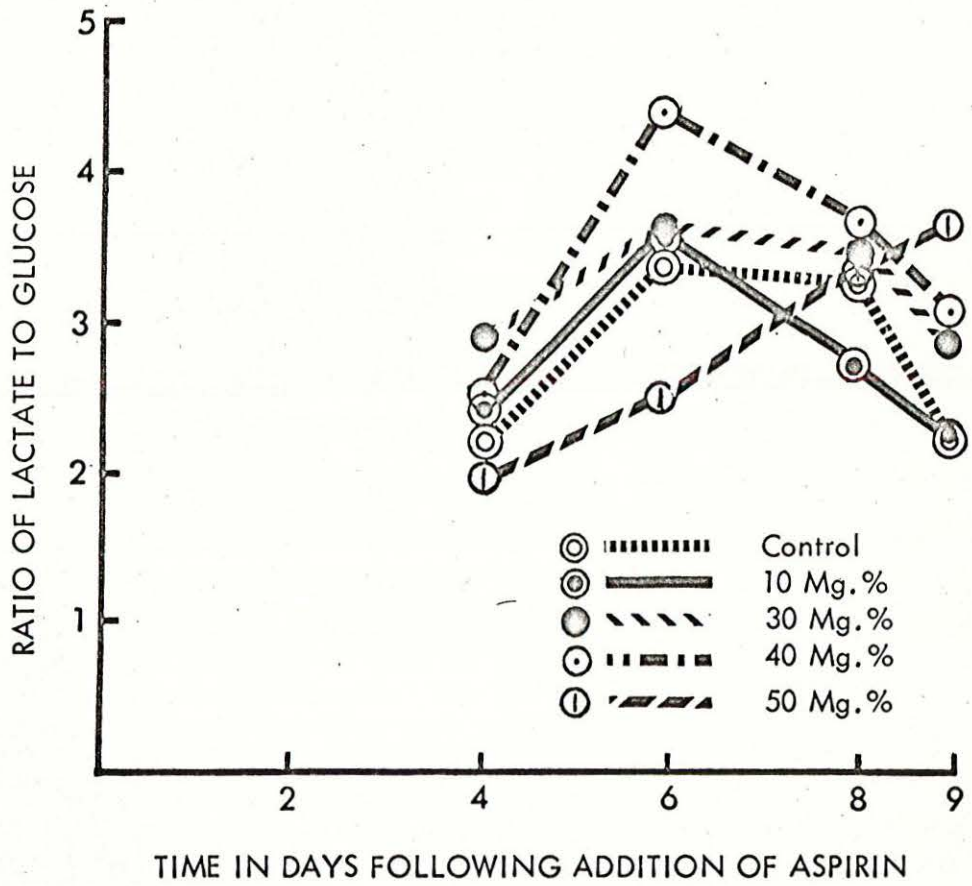
TABLE I

The effects of various concentrations of aspirin upon growth, glucose utilization, and lactate production in Low Line cells.

Time of Exposure in Days	Experimental Conditions	No. of cells/ml.	Glucose Utilized ($X10^{-6}$mg./ml.)	Lactate Produced ($X10^{-6}$mg./ml.)	Glucose / Lactate
4	control	895,000	0.953	2.105	2.209
	10 mg%	1,015,000	0.732	1.778	2.429
	30 mg%	780,000	1.093	3.182	2.911
	40 mg%	792,500	0.861	2.182	2.534
	50 mg%	585,000	1.166	2.316	1.986
6	control	1,427,500	0.621	2.092	3.369
	10 mg%	1,437,500	0.593	2.104	3.548
	30 mg%	1,002,500	0.900	3.279	3.643
	40 mg%	980,000	0.836	3.670	4.390
	50 mg%	717,500	1.095	2.776	2.535
8	control	1,597,500	0.524	1.710	3.263
	10 mg%	1,845,000	0.417	1.136	2.724
	30 mg%	1,192,500	0.587	2.050	3.492
	40 mg%	1,087,500	0.683	2.543	3.723
	50 mg%	570,000	1.303	4.414	3.388
9	control	1,665,000	0.566	1.258	2.223
	10 mg%	1,820,000	0.523	1.188	2.272
	30 mg%	1,125,000	0.817	2.363	2.892
	40 mg%	950,000	0.976	3.027	3.101
	50 mg%	482,500	1.715	6.344	3.699

FIGURE 5. Ratios of lactate produced to glucose utilized in Low Line cells exposed to various concentrations of aspirin.

FIGURE 5


RATIO OF LACTATE TO GLUCOSE
5
4
3
2
1
2
4
6
8
9
TIME IN DAYS FOLLOWING ADDITION OF ASPIRIN
Control
10 Mg.%
30 Mg.%
40 Mg.%
50 Mg.%

concentrations of aspirin were of greater magnitude than those of the control cells, with a direct relationship between concentration and ratio. It should be noted that while cells exposed to 10 mg.% aspirin fell into the general pattern of those exposed to higher concentrations, glucose utilization at that concentration appeared to be inhibited rather than increased as it was for the others.

Fat Determinations

Low Line cells were cultured on slides in Leighton tubes in order to ascertain fat content by means of the Oil-Red-O technique. Concentrations of 50 mg., 30 mg., and 10 mg.% aspirin were used.

A definite increase in fat was seen at the highest concentration on the fifth day of exposure, but the effect at lower concentrations (if any) was difficult to determine.

The Long-term Effects of Aspirin

Low Line cells were used for a further study of the long-term effects when subjected to 10 mg.% aspirin, the concentration which had previously been found to stimulate proliferation. Cells were cultured in T-60 flasks; the medium was changed every two days. When growth became heavy, the cultures were gently shaken and the cells that became detached were discarded, resulting in a much lower population of cells. This was necessary to insure further possible proliferation while exposed to the experimental conditions.

These cultures were carried for approximately one month, and although

most cells appeared normal during that period, there was a considerable increase in the number of "giant" cells present. Toward the end of the experiment, their number stabilized. A photomicrograph of cells selected at random is shown in Figure 6. It is interesting to note that several of the "giant" cells are adjacent to each other. This may be significant because these cells are thought to undergo nuclear division only, while here it appears that they may have undergone cytoplasmic division; the possibility also exists that a factor which causes alteration to occur in one cell also may affect nearby cells.

This experiment has been repeated using transformed K cells. Gentle shaking was again used to thin the cultures; however, they were occasionally scraped and subcultured. At 30 days no significant increase in the number of "giant" cells in the experimental cultures was noted.

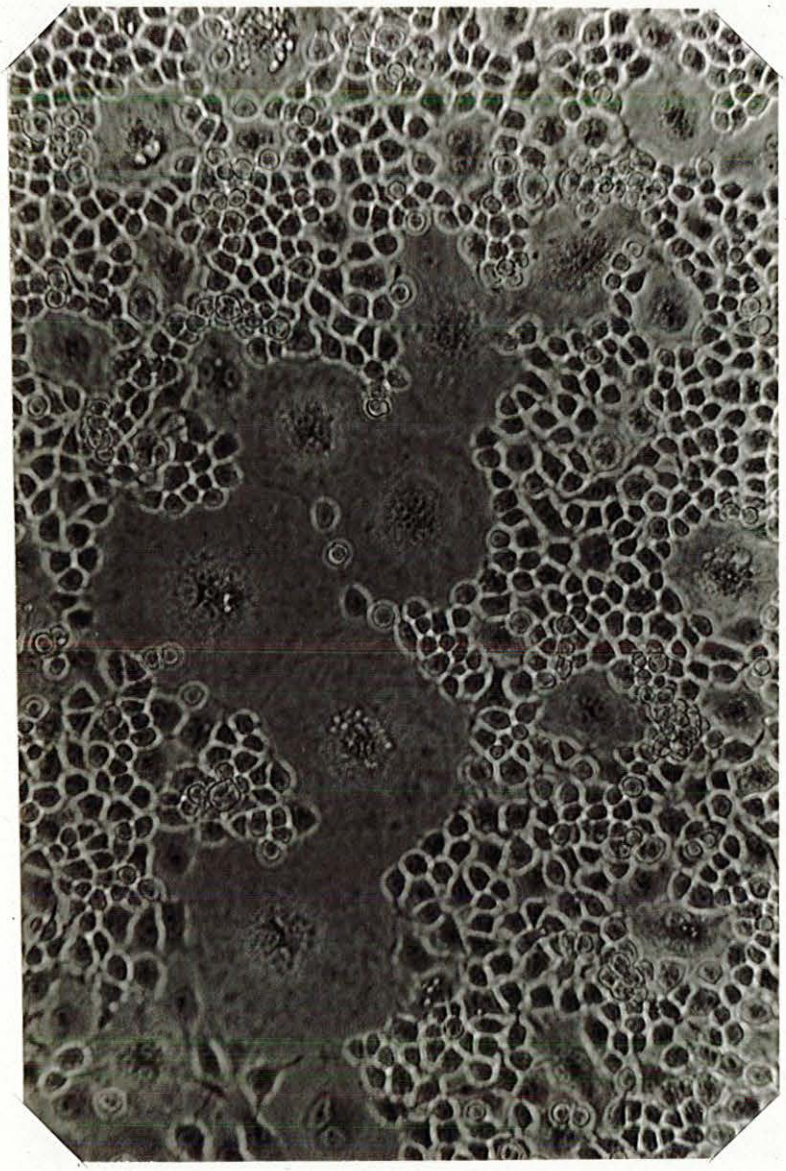
Time-lapse Studies

A cine-photomicrographic time-lapse study was made with Low Line cells in a normal culture in order to obtain a better understanding of the behaviour of the "giant" cells and their relationship to this study. Exposures were made at the rate of 1 frame per 15 seconds for a period of 18 hours.

An interesting phenomenon was noted when analyzing the film after developing: a so-called "giant" cell with a single nucleus was seen to coalesce with a cell of normal morphology to form a bi-nucleate giant cell (Figure 7). This process may be related to the formation of the multi-nucleate giant cells observed in the long-term experiment and thus merits further study.

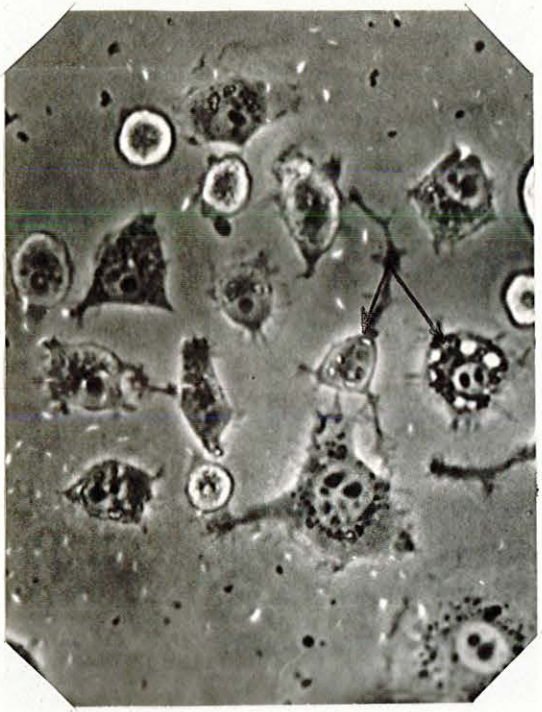
FIGURE 6. A photomicrograph of a Low Line culture showing the numerous "giant" cells that have appeared after approximately 30 days exposure to a 10 mg.% concentration of aspirin.

FIGURE 6

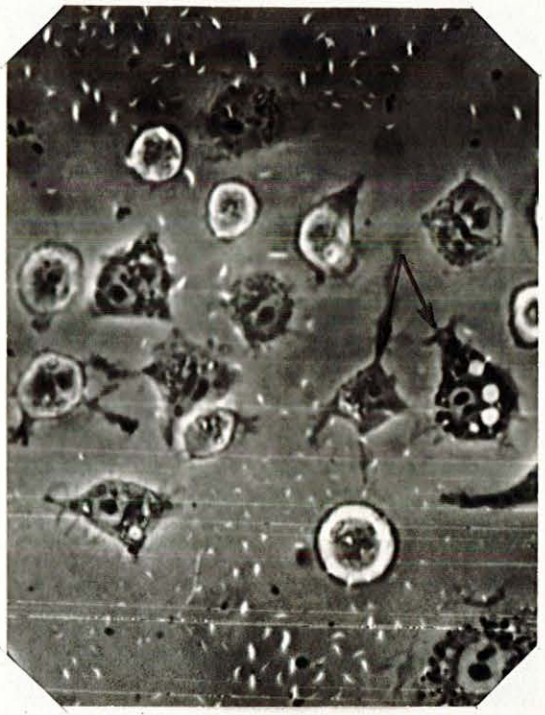


FIGURES 7 A-D. Photomicrographs taken from four widely separated frames of a 16 mm film used in a time-lapse study made in an attempt to obtain information pertaining to the formation of "giant" cells in culture. A single nucleate "giant" cell and a cell of normal morphology coalesce to form a binucleate "giant" cell. Arrows indicate the two cells involved.

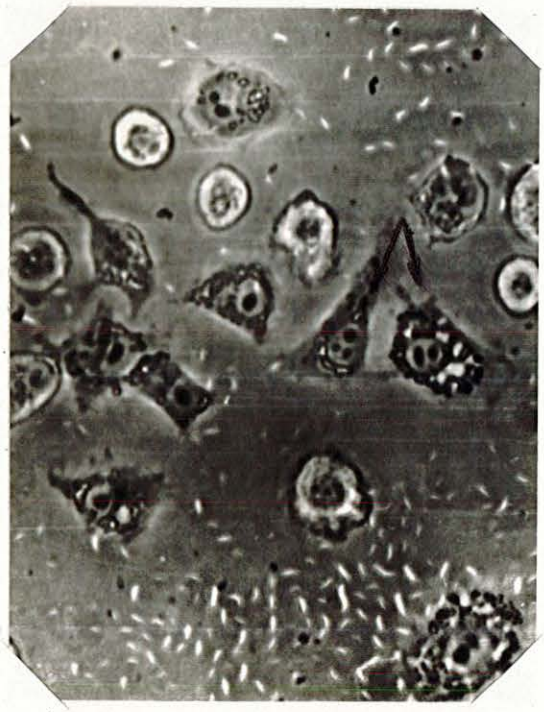
FIGURE 7



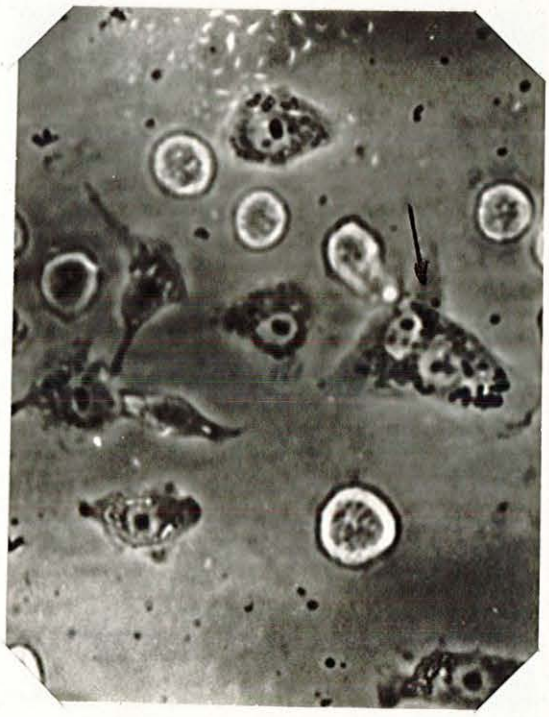
A



B



C



D

A time-lapse study was also made of a culture of K cells that were exposed to a concentration of 75 mg.% aspirin. Eight hours after the beginning of the filming sequence, the aspirin was introduced by means of a syringe with a millipore adaptor. The cells were then observed at various magnifications for a period of two days. Exposure was at the rate of one frame per minute. No significant change in the cells could be detected during the period of observation.

Cytochrome Oxidase

Cytochrome oxidase catalyzes the reaction:

$$2 \text{ ferricyto C} + 2 H^+ + 1/2\ O_2 \qquad 2 \text{ ferricyto C} + H_2O$$

The effects of aspirin on this enzyme were studied using Low Line cells cultured on slides in Leighton tubes. Concentrations of 50, 30, and 10 mg.% were used. After six days exposure, the slides were stained for cytochrome oxidase activity. No significant differences could be detected.

Succinic Dehydrogenase

As mentioned above, several of the dehydrogenases are thought to be inhibited by members of the salicylate family. For this reason, the effect of aspirin on the enzyme, succinic dehydrogenase, was investigated.

K cells were grown on slides in Leighton tubes and exposed to concentrations of 50 and 10 mg.% aspirin for a period of eleven days. At the end of this period, the degree of succinic dehydrogenase activity in the cells was ascertained.

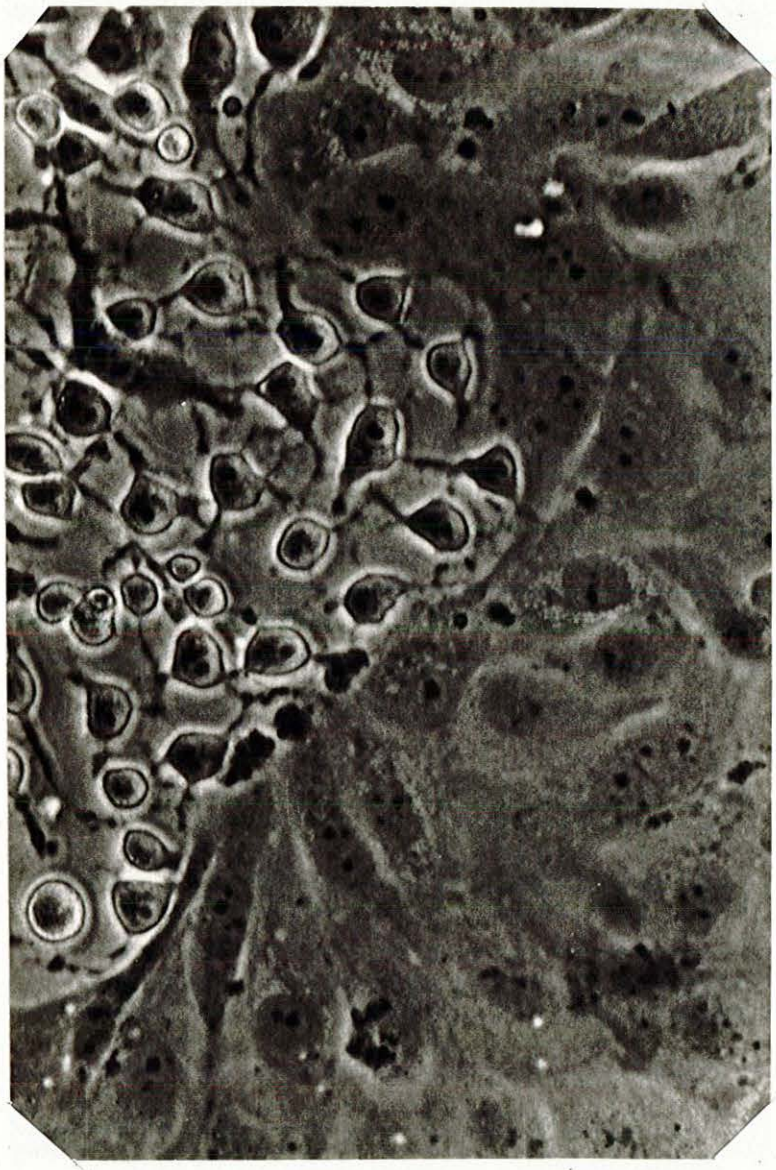
As compared to the controls, cells in both 50 and 10 mg.% showed a decrease in activity. The decrease was somewhat greater in those subjected to 50 mg.%.

Differences in Growth Inhibition Between Transformed and Non-transformed Cells Exposed to Aspirin.

In the preliminary studies on the effects of aspirin, it was discovered that cytotoxic effects developed earlier and were much more pronounced in Low Line than in K cells. This posed a problem as to whether this effect was due to a species difference or to the fact that one type of cell is transformed while the other is not. It is of interest to note that during one experimental run the diploid K cells began to undergo transformation. This was quite noticeable because of a great increase in proliferation rate, chromosome number (octoploid), and a change in general morphology. This transformation took place most readily in the control cultures and in the cultures of low aspirin concentration but was distinctly inhibited in the 50 mg.% aspirin. Later growth studies of transformed K cells (described above) confirmed that they were, indeed, more susceptible to the cytotoxic effect of aspirin. A photomicrograph of a culture of K cells undergoing transformation is shown in Figure 8.

FIGURE 8. A photomicrograph of a normal culture of K strain cells undergoing transformation. The transformed cells are at the left.

FIGURE 8



DISCUSSION

DISCUSSION

In this study of the effects of acetylsalicylic acid at the cellular and sub-cellular level, concentrations from 10 mg.% to 200 mg.% were used. It was found that concentrations of 30 mg.% or more were toxic and retarded growth. These effects increased in magnitude with increasing concentration. While this was true for all three cell types used, some appeared to be more susceptible than others. At the lowest concentration used (10 mg.%) no toxic signs were observed and growth was actually stimulated in the Low Line cells. Wilhelmi (1963) has reviewed the literature and concluded, with the aid of evidence from his own work, that salicylates can be either acceleratory or inhibitory on tissue regenerative processes depending on dosage, mode of administration, experimental conditions, and species. In general, the results obtained in this part of the study tend to support his observations.

The initial concentrations of acetylsalicylate used, with the possible exception of the highest, are comparable to the plasma salicylate levels encountered in therapeutic situations. Here, it should be kept in mind that as acetylsalicylate dissociates to salicylate, the concentration of the latter is reduced.

The effect of varying concentrations of aspirin on glucose utilization and lactate production in Low Line cells was also studied. Here, the control cultures showed a high rate of glucose uptake during the early part of the logarithmic growth phase. The rate declined rapidly at first and more slowly later in the phase. A reversal of this trend took place as the cells became

crowded in the cultures. In general, this sequence of events follows the pattern set by other cells in vitro as described by Fulton et al (1956), Bryant et al (1958), and Munyon and Merchant (1959), in their studies of glucose utilization in relationship to growth and cell density.

Cells exposed to the various concentrations of aspirin showed marked differences in glucose utilization. Two factors must be considered here: the first is the indirect effect that aspirin has on utilization through its effect on growth and cell density; the second is the more direct biochemical influence.

Considering the effect on cell growth, it was found that cells exposed to a medium containing 10 mg.% aspirin had a higher rate of cell proliferation than those of the control cultures, with a concurrent decrease in glucose utilization per cell at any particular period. The opposite was true in the case of the higher concentrations of aspirin; proliferation rates were lower and glucose utilization at each particular period was higher. All experimental cells showed a decrease in the amount of glucose utilized during the log phase of growth with a reversal during the decline phase. In short, they appeared to be following a pattern dictated by the growth and density effect; however, a closer inspection shows that another factor is in operation. This is readily apparent in the case of the highest concentration. Here, the minimum amount of glucose utilized exceeds that utilized by any other concentration at any time during its growth cycle. Two known biochemical effects of aspirin suggest an explanation. As mentioned earlier, aspirin is known to inhibit the action of many, if not all, of the dehydrogenases that require either a flavin or a pyridine nucleo-

tide as a co-factor. Salicylate, a hydrolysis product of aspirin, is also known to uncouple oxidative phosphorylation; therefore, aspirin can act in at least two ways to decrease the efficiency of utilization of glucose in the formation of adenosine triphosphate. When considered as a dehydrogenase inhibitor, aspirin can affect several of the reactions involved in the degradation of glucose, both during glycolysis and in the citric acid cycle. In glycolysis the phosphotriose dehydrogenase, used in conversion of glyceraldehyde-3, 1-diphosphate, is the first possible point of interference. The second possible point would be at the pyruvate dehydrogenase complex as pyruvate is funneled into the citric acid cycle. During the actual cycle, four enzymes are susceptible; they are the isocitric dehydrogenase, the dehydrogenase involved in the conversion of alpha-ketoglutarate to succinate, the succinic dehydrogenase (as demonstrated in this study), and the malic dehydrogenase. Since the main effect of aspirin in this respect is on the citric acid cycle, it seems probable that the cell would become more dependent on the less efficient glycolytic form of respiration which would, in effect, require an increased glucose uptake.

As mentioned earlier, it has been found by Brody (1956) and others that salicylates are capable of uncoupling oxidative phosphorylations along the whole respiratory chain. This indicates that those hydrogens made available for respiratory assembly may also be utilized in an inefficient manner, putting on a still greater demand for glucose. Considering all these factors, the increase in glucose utilization observed at the higher concentrations is not surprising. In order to get a better idea of what is actually happening, however, the magnitude

of the effect at each of these particular points of interference should be studied.

Closely associated with glucose utilization is lactate production. This study has shown a trend toward a direct relationship between the two at the various concentrations tested. When the relationship between the concentrations was considered, it was discovered that, in general, the higher concentrations demonstrated increasingly higher lactate to glucose ratios. Respirations seemed to be shifting toward a more glycolytic mode. Here again, the effect of aspirin on the dehydrogenases involved in the citric acid cycle affords a possible explanation.

Dawkins et al (1967) have shown that aspirin inhiblts the action of lactate dehydrogenase and, therefore, it has an even more direct influence on the situation.

When considering glucose utilization and lactate production in general one should keep in mind the possibility of utilization of lactate by the cell; this has been shown to occur under certain conditions by Munyon and Merchant (1959) and Graff et al (1965). If this phenomenon is taking place to any appreciable extent it could easily have significant effects on the results of this type of study.

Related to glucose and lactate metabolism is the metabolism of fats. In this study it has been shown that aspirin, at high concentrations, caused an increase in the amount of fat present within the cells. The effects on the dehydrogenases probably play a significant role here, also.

The production of the long-chain fatty acids necessary for the formation of fats and other lipid materials can take place by either of two known pathways within the cell. The first one is restricted to the mitochondria and consists of

elongating already existing fatty acids by condensations with acetyl-CoA. The second pathway involves a succession of condensations starting with acetyl-CoA and malonyl-CoA, which is the product of carboxylation of acetyl-CoA. While acetyl-CoA is produced in the mitochondria, it does not readily diffuse outward to the cytoplasm. For this reason, a source of cytoplasmic acetyl-CoA is necessary if the second pathway is to operate. One good source of this coenzyme makes use of citrate produced in the mitochondria during the operation of the citric acid cycle. This citrate, after diffusion to the cytoplasm, reacts with coenzyme A and ATP to form the necessary acetyl-CoA.

In the citric acid cycle an equilibrium exists between citrate, cis-aconitate, and isocitrate. If aspirin inhibits the dehydrogenation of isocitrate, there will be an accumulation of citrate which may diffuse into the cytoplasm and become available for fatty acid synthesis.

Beside the possibility that more citrate may be made available for cytoplasmic fatty acid snythesis, aspirin may also interfere with their mitochondrial oxidation. Of the two oxidations that occur during each cycle, one requires FAD and the other requires NAD^+; they are therefore susceptible to the action of aspirin.

When the overall scheme is considered, it can be seen that a build-up of fats might be expected due to increased availability of citrate for the formation of the acetyl CoA and malonyl CoA necessary for the synthesis of fatty acids as well as the inhibition of the oxidation of fatty acids.

In this study it was shown that aspirin, in concentrations as high as

50 mg.%, had no apparent effect on the enzyme, cytochrome oxidase; however, Hines, Bryant, and Smith (1963), using guinea pig liver preparations, have shown that salicylate, in a concentration of 140 mg.%, is capable of reducing this enzyme's activity.

Previous work concerning the effects of salicylate upon succinic dehydrogenase activity has resulted in conflicting results. Kaplan, Kennedy, and Davis (1954) have reported that a concentration of approximately 90 mg.% salicylate was capable of inhibiting the activity of the enzyme in rat kidney and liver homogenates. Later, Bryant, Smith, and Hines (1963), using rat mitochondrial preparations, claimed that concentrations up to 140 mg.% had no effect on activity and that the earlier results of Kaplan et al were in error because, in the relatively complex system they used, the apparent effect on succinic dehydrogenase could have, in reality, been due to inhibition of malate dehydrogenase.

The work presented here has indicated that aspirin, in concentrations as low as 10 mg.%, is capable of inhibiting succinic dehydrogenase activity. Whether or not this is a characteristic peculiar to acetylsalicylate remains to be determined.

While relatively little is known about 'giant' cells in culture, it is generally thought that they are, in some way, related to adverse environmental conditions; however, because of certain similarities between them and the macrophages observed in vivo, it was thought that their study might yield some information about the inflammatory response.

It has been found that the 'giant' cells do increase in numbers in cultures exposed to aspirin, and that this is true even when no toxic symptoms are evident, as demonstrated in the case of long-term exposure to a 10 mg.% concentration.

Little information concerning their multi-nucleate condition is also available. Once, it was generally thought that this was the result of nuclear division without cytokinesis. While the time-lapse study described here does not rule out that possibility, it does demonstrate that the multi-nuclear condition may also result from cell fusion.

At this time, not a great deal can be said concerning the observed differences in toxicity between K strain and transformed K cells. When one considers the fact that Low Line cells are also more susceptible to the toxic effects of aspirin, it seems possible that this greater effect may be a characteristic of transformed cells in general, and may be related to their higher metabolic rate; however, further investigations into this area are necessary before any firm conclusions can be drawn.

SUMMARY

SUMMARY

1. The effects of aspirin upon the growth of K strain, transformed K, and Low Line cells grown in culture were studied. Low Line cells were used to investigate the effects of aspirin on glucose uptake, lactate production, fat deposition, and cytochrome oxidase activity. The long term effects were observed in both Low Line and transformed K cells. Time-lapse studies of "giant" cells in control cultures (Low Line) were also made. In addition, the effect upon succinic dehydrogenase activity in K strain cells was observed.

2. Aspirin, at a concentration of 10 mg.%, stimulated growth in Low Line cells; concentrations of 30 mg.% and above proved to be increasingly inhibitory. Of the two concentrations used in the growth studies of transformed K cells, the 50 mg.% was inhibitory while the effect of the 10 mg.%, if any, was difficult to determine. K strain cells appeared to be more resistant to the toxic effects than either Low Line or transformed K cells.

3. High concentrations of aspirin appeared to either inhibit cell transformation or reduce the rate of proliferation of transformed cells in cultures of K strain.

4. In Low Line cells, a concentration of 10 mg.% aspirin led to a decrease in glucose utilization and lactate production as compared to cells in the control cultures, while higher concentrations led to increasingly higher

rates. The ratios of lactate produced to glucose utilized were, in general, higher than those of the controls, with a trend toward a direct relationship between concentration and effect.

5. The deposition of fats in Low Line cells was increased when the cells were exposed to a concentration of 50 mg.% aspirin for several days. Little, if any, effect was noted at the lower concentrations.

6. Low Line cells exposed to a concentration of 10 mg.% aspirin for approximately one month showed no perceptible changes in morphology; however, the number of "giant" cells present increased greatly during that time. Transformed K cells showed no appreciable change in morphology or in the number of "giant" cells when exposed to 10 mg.%.

7. Aspirin, at the concentrations used, did not affect cytochrome oxidase activity in Low Line cells.

8. Aspirin was shown to inhibit succinic dehydrogenase activity in K strain cells at the concentrations used. There was a direct relationship between concentration and degree of inhibition.

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