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A comparison of the effects of aspirin, sodium salicylate, and acetate on cells in vitro

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A COMPARISON OF THE EFFECTS OF ASPIRIN, SODIUM SALICYLATE, AND ACETATE ON CELLS IN VITRO

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by

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A COMPARISON OF THE EFFECTS OF ASPIRIN, SODIUM SALICYLATE, AND ACETATE ON CELLS *IN VITRO*

Abstract of Dissertation

Salicylic acid and its congeners have long been used for the management of pain, fever, and inflammation. Of this group, acetylsalicylic acid, or aspirin (ASA) has proved to be the most potent in each case. Although hydrolysis, the first step in biotransformation of aspirin takes place very rapidly, many workers feel that the greater effectiveness is due to a unique action of the intact molecule; however, others disagree. In this study, the effects of aspirin and its hydrolysis products, salicylate and acetate, on mammalian cells *in vitro* were compared in order to obtain a greater amount of information relating to the problem.

Various concentrations of aspirin, sodium salicylate, the combination of sodium salicylate and sodium acetate, and sodium acetate by itself, were used. The effects on growth, glucose utilization, lactic acid production and fat deposition were ascertained. At 0.55mM, the compounds exerted little or no consistent effect on growth, glucose utilization or lactic acid production. While some effect could be seen at 1.67mM, a clear-cut trend emerged at 2.87mM. Aspirin, at that concentration, was most inhibitory to growth and resulted in the greatest amount of glucose utilized and lactic acid produced. Inhibition of growth and increase in glucose utilization and lactic acid production were less marked on exposure to sodium salicylate than in the case of aspirin and the combination of salicylate and acetate resulted in a still further decrease in effect. Sodium acetate stimulated growth and resulted in glucose utilization and lactic acid production similar or slightly higher in degree than in the controls. The highest ratio of lactate produced to glucose utilized resulted from exposure to aspirin. Decreasing ratios were encountered for sodium salicylate and the combination of salicylate and acetate. Ratios in the case of acetate were similar or slightly lower than those of the control cultures. Variation in fat content appeared to be related, at least in part, to factors other than the compounds under investigation, thus making interpretation difficult.

Results of this study indicated that the hydrolysis products of aspirin did not act together to produce an effect similar in magnitude to aspirin on growth, glucose utilization or lactic acid production; however, this investigation as well as many others has demonstrated that sodium salicylate has similar effects to aspirin on a variety of phenomena but almost invariably to a lesser extent when used at the same concentration. This, when considered with other factors, such as relative degree of protein binding, suggests the possibility that aspirin is unique by virtue of its ability to get to the site of action but is not necessarily unique otherwise.

It was proposed that the antagonistic action of acetate on salicylate may be due to an ability of acetate to bypass the pyruvate dehydrogenase complex on entrance into the citric acid cycle. This suggests that the effect of salicylate on this complex is of particular significance.

ACKNOWLEDGEMENTS

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Finally, words cannot express the gratitude he feels for his father, the late Dr. Alexandre Barron, who made his education possible.

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INTRODUCTION

INTRODUCTION

Aspirin, or acetylsalicylic acid, is probably one of the most widely used drugs in existence. It belongs to a group of agents which have the 2-hydroxybenzoate radical in common and are collectively known as the salicylates. Naturally occurring members of this group are found in the bark of willow, flowers of meadowsweet, and oil of wintergreen and, in their natural forms they have been used as medicaments since very early times.

While salicylic acid had been prepared from plant sources several years earlier, it was not until 1852 that Gerland first synthesized it; C. F. Gerhardt was successful in synthesizing acetylsalicylic acid in 1853. The process was quite difficult, however, and it was not until 1893 that Felix Hoffman, A Bayer chemist, developed a practical method for producing large quantities of what was later to be called aspirin.

The salicylates have proven to be remarkably effective agents in the management of pain, inflammation, and fever and for this reason they have been the subject of considerable research.

One of the earliest discoveries concerning metabolic effects of these compounds was made by Singer (1901), who noted that toxic doses of acetylsalicylic acid, given to rabbits, caused an increase in oxygen consumption. While an extensive amount of work related to this phenomenon was done during the following years, it was not until much later that it was studied in an *in vitro* situation. Sproull (1954)

demonstrated that concentrations of 5 to 30 mg.% salicylate caused an increase in oxygen consumption in mouse liver preparations, and Brody (1956) further defined the site of this action by demonstrating the same phenomenon using mitochondrial preparations of rat liver and kidney. The increased oxygen consumption appears to be caused by the uncoupling of oxidative phosphorylation. Although phosphorylations associated with the entire respiratory chain are affected, Peniall (1958) has presented evidence that the final one is the most sensitive to salicylate.

In addition to the uncoupling action, salicylates also act to reduce cellular ATP levels by stimulating adenosene triphosphatase activity as demonstrated by Brody (1956) and Falcone (1959).

Increased oxygen consumption due to salicylate is well documented, however, Fishgold, Field, and Hall (1951), using rat cerebral cortex preparations, have shown that high concentrations of salicylate cause a decrease in consumption. This appears to be due to an interference with the oxidation of intermediary metabolites. Evidence to support this has come from the work of Kaplan and Kennedy (1954). Using homogenates of rat liver and kidney, they found that concentrations of approximately 90 mg.% inhibit α-keto glutarate dehydrogenase and succinic dehydrogenase. Hines, Bryant, and Smith (1963) employed lysed guinea pig mitochondria and indicated that the apparent succinate dehydrogenase inhibition is actually due to inhibition of malate dehydrogenase.

Smith, Bryant, and Hines (1964), using preparations of respiring mitochondria, have further shown that the inhibition of malate dehydrogenase is due to reversible salicylate competition for the pyridine nucleotide coenzyme.

It is now generally known that any dehydrogenase that operates with either a flavin or pyridine cofactor is susceptible to salicylate inhibition.

Another important effect of salicylate is that exerted on glutamate metabolism. Research in this area by several investigators has indicated that practically every enzyme system using glutamate as a substrate is inhibited *in vitro* by salicylate. Of these, the inhibition of transaminations appears to be the most significant.

In rat serum containing 5 mM salicylate, Huggins, Smith, and Moses (1961) have demonstrated inhibition of glutamic-pyruvic transaminase activity. At the same concentration, Yoshida, Metcoff, and Kaiser (1961) observed inhibition in homogenates of rat brain, kidney, liver, and muscle. Later, Gould and Smith (1965) presented evidence that concentrations of 10 mM salicylate caused a general inhibition of transaminations involving glutamate. They further demonstrated that this inhibition was due to a competition with the amino acid and the α-oxoacid substrates.

Other effects of salicylate on glutamate metabolism are also significant. Smith and Smith (1966) in a summary of specific actions demonstrated *in vitro*, list its conversion to glutamine, decarboxylation to γ-aminobutyrate, incorporation into proteins, conversion to proline, and oxidative deamination to α-oxoglutarate in addition to the inhibition of transaminations already mentioned. They also suggest how these actions may relate to *in vivo* situations. For example, inhibition of the conversion of glutamate to glutamine may affect the synthesis of mucopolysaccharides and purine nucleotides. Interference with the formation of γ-aminobutyrate in brain

tissue may be the cause of the convulsions due to toxic levels of salicylate, while inhibition of related protein synthesis can have several effects including interference with connective tissue metabolism. In addition, the effect on transaminase activity probably causes significant alterations in the relative size of the various amino acid pools, thereby upsetting normal metabolic patterns. This appears to be related to the "overflow" aminoaciduria and associated negative nitrogen balance encountered at high salicylate concentrations.

Many, if not all of the reported effects of salicylates may in some way be related to the uncoupling of oxidative phosphorylation, activation of ATP-ase, inhibition of the dehydrogenases and/or transaminase inhibition. The following several examples are representative.

Manchester et al (1953), have shown that salicylate inhibits the in vitro incorporation of amino acids into protein in rat diaphragm.

Bellamy, Huggins, and Smith (1963), obtained results indicating that salicylate inhibits the biogenesis of some forms of collagen and collagen-like proteins in rat costal cartilage. Bostrom et al (1963), using bovine heart valves, and Whitehouse et al (1961, 1962, 1963, 1964) using bovine cartilage and cornea as well as rat cartilage, observed an inhibition in the synthesis of mucopolysaccharide sulfates in vitro. Bostrom et al (1964) have also shown an in vivo inhibition in rat rib cartilage.

The effects of salicylate on fat metabolism are contradictory. Brody (1956) with preparations of rat liver mitochondria, has shown that a 1mM concentration depressed the oxidation of octanoate by over

50%; however, Whitehouse (1963) had demonstrated an increase in oxidation of octanoate in cartilage slices at a concentration of 4 mM, but no effect at 5 mM. Barron (1970) has indicated that a concentration of 2.78 mM aspirin caused an increase in fat deposition in cultured mouse fibroblasts which, if due to inhibition of oxidation rather than stimulation of synthesis, would tend to add support to Brody's work.

The recent discovery that salicylate as well as other "anti-defensive" anti-inflammatory agents inhibit the production of certain prostaglandins has proven to be a significant step toward clarifying their mode of action. For example, Vane (1971) has reported that both aspirin and sodium salicylate blocked the synthesis of prostaglandins E_2 and F_{2a} in cell-free homogenates of guinea pig lung, with aspirin being the more potent of the two. Smith and Willis (1971) have reported similar results using a suspension of human platelets.

Since the prostaglandins may play a role in the onset of fever and certain types of pain as well as inflammation, many investigations are now being carried on in this area.

In the brief review presented above, it can be seen that while a variety of experimental systems have been utilized, little work has been done concerning the effects of salicylate on cells in culture. The relative newness of this technique is the probable reason; however, it offers many advantages. It offers a large, homogeneous population in a state that lends itself well to a great variety of cytological procedures as well as to detailed microscopic examination. The system is also free of the influence of other cell types, tissues,

and organs but still retains the quality of being intact. While culture conditions must be carefully controlled, it is still a relatively simple matter to provide an environment with few variables which is extremely well suited for experimentation.

Using cell culture techniques, Barron (1970) has studied the effects of aspirin on growth, glucose utilization and lactate production; also its effects upon the enzymes succinic dehydrogenase and cytochrome oxidase, as well as the effect on fat deposition already mentioned.

Growth in Low Line cells (derived from mouse subcutaneous connective tissue), as indicated by increase in cell number, was stimulated by a concentration of 0.55 mM aspirin; it was inhibited by concentrations of 1.67 mM and 2.78 mM.

Glucose utilization and lactate production decreased when the cells were exposed to a concentration of 0.55 mM aspirin but in higher concentrations glucose utilization and lactate production increased. The ratios of lactate produced to glucose utilized were increased in all cases with a direct relationship between concentration and effect.

Aspirin did not affect cytochrome oxidase activity in Low Line cells at concentrations as high as 2.78 mM: succinic dehydrogenase activity was inhibited in K strain (adult rat kangaroo kidney) cells exposed to concentrations as low as 0.55 mM. In addition, it was noted that long term exposure (30 days) of Low Line cells to a concentration of 0.55 mM aspirin caused an unexplained increase in the number of "giant" cells.

It is obvious that since Singer's time a great deal has been learned about the metabolic effects of aspirin and salicylate in general. Unfortunately, this information does not give us any clear

cut indication how the anti-inflammatory, analgesic, or anti-pyretic actions are achieved. Although the mechanisms are undoubtedly complicated, certain discoveries, such as their effect on the prostaglandins, have helped considerably to clarify the picture; however, many questions remain to be answered. One relates to the fact that aspirin acts in a different manner than other salicylates. Thompkins and Lee (1969) have shown that the intact aspirin molecule does not uncouple oxidative phosphorylation as does salicylate. In addition, as already noted, it is a more potent inhibitor of certain prostaglandins. Whether or not these actions are totally or in part related to its greater potency as an anti-inflammatory and anti-pyretic agent remains to be seen.

Because relatively few reports appearing in the literature have dealt with the effects of salicylate at the cellular level (with little or nothing reported on growth, glucose utilization, lactic acid production or fat deposition) and because little is known concerning aspirin's unique effects, the following investigations were made.

MATERIALS AND METHODS

MATERIALS AND METHODS

Origin and Nature of the Cells Used

The cells used for this study are commonly known as Low Line or NCTC clone 2555 (American Type Culture Association number is CCL12). This clone was derived from a culture of subcutaneous areolar and adipose connective tissue taken from a normal 82 day old C3H/HeN mouse by Likely, Sanford, and Earle (1952). Sanford, Likely, and Earle (1954) later found that injection of cells of this parent strain into mice produced sarcomas in about one percent of the cases while, under similar conditions, cells from one of the clones developed from this line (clone 2555) produced no sarcomas; however the term "Low Line", which originally denoted low tumor producing fibroblasts, is still used.

These cells have been cultured since their origin in medium NCTC 109 (now identical to NCTC 135) plus 10% newborn calf serum. The contents of the medium are shown in Table I, page 10.

Nature of the Chemical Agents

In the experiments reported here, the cells were exposed to acetylsalicylic acid (aspirin), sodium salicylate, sodium acetate, or a combination of sodium salicylate and sodium acetate.

Acetylsalicylic acid (USP) exists mainly in the form of elongated monoclinic crystals. It is colorless and odorless with a solubility in water of 250 mg. per ml. at 20°C. Its chemical formula

TABLE I

The Contents of Medium NCTC 135

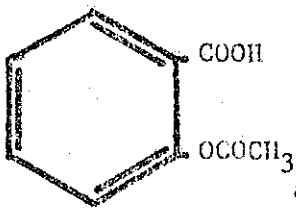
NCTC 135 MEDIUM

Ref.: Evans, V. J., et al., Ca. Res., 16:77-94 (1956).
McQuilkin, W. T., et al., J. Nat. Ca. Inst., 19:885 (1957).
Evans, V. J., Bryant, J. C., Kerr, H. A., and Schilling, E. L., Exp. Cell Res., 36:439 (1964).

Component	mg/L	Component	mg/L
NaCl	6800.0000	Pyridoxal HCl	0.0625
KCl	400.0000	Niacin	0.0625
$CaCl_2$ (anhyd.)	200.0000	Niacinamide	0.0625
$MgSO_4$	100.0000	D-Ca pantothenate	0.0250
$NaH_2PO_4 \cdot H_2O$	140.0000	Biotin	0.0250
D-Glucose	1000.0000	Folic acid	0.0250
L-Alanine	31.5000	Choline Cl	1.2500
L-Alpha-Amino-n-butyric acid	5.5000	Vitamin B_{12}	10.0000
L-Arginine HCl	31.2000	i-Inositol	0.1250
L-Asparagine $\cdot H_2O$	9.2000	Para-Aminobenzoic acid	0.1250
L-Aspartic acid	9.9000	Vitamin A	0.2500
L-Cystine	10.5000	Calciferol	0.2500
D-Glucosamine HCl	3.9000	Menadione	0.0250
L-Glutamic acid	8.3000	Disodium alpha-tocopherol phosphate	0.0250
L-Glutamine	135.7000	Sodium glutathione	10.0000
Glycine	13.5000	Ascorbic acid	50.0000
L-Histidine HCl $\cdot H_2O$	26.7000	Diphosphopyridine nucleotide	7.0000
Hydroxy-L-proline	4.1000	Triphosphopyridine nucleotide (mono sodium)	1.0000
L-Isoleucine	18.0000	Coenzyme A	2.5000
L-Leucine	20.4000	Cocarboxylase	1.0000
L-Lysine HCl	38.4000	Flavin adenine dinucleotide	1.0000
L-Methionine	4.4000	Sodium uridine triphosphate	1.0000
L-Ornithine HCl	9.4000	Deoxyadenosine	10.0000
L-Phenylalanine	16.5000	Deoxyguanosine	10.0000
L-Proline	6.1000	Thymidine	10.0000
L-Serine	10.5000	5-Methylcytosine	0.1000
L-Taurine	4.2000	Tween® 80*	12.5000
L-Threonine	18.9000	D-Glucuronolactone	1.8000
L-Tryptophan	17.5000	Sodium glucuronate $\cdot H_2O$	1.8000
L-Tyrosine	16.4000	Sodium acetate $\cdot 3H_2O$	50.0000
L-Valine	25.0000	Ethanol - For solublizing lipid components	40.0000
Thiamine HCl	0.0250	Phenol red	20.0000
Riboflavin	0.0250	$NaHCO_3$	2200.0000
Pyridoxine HCl	0.0625		

* *Trademark of Atlas Powder Co.*

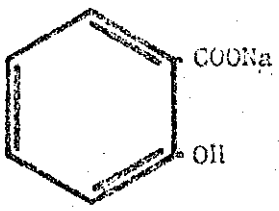
is $CH_3COOC_6H_4COOH$ with a molecular weight of 180.15:



acetylsalicylic acid

Although aspirin is stable when dry, exposure to moist air results in a gradual hydrolysis to acetic acid and salicylic acid. For this reason, care was taken to keep the container tightly closed and in a dessicator when not in use. Because of its instability in solution, aspirin was added to the medium immediately before each medium change.

Sodium salicylate (USP) is found in the form of white, odorless crystals, scales, or powder. It has a solubility of one gram in 0.9 ml. of water and the molecular weight is 160.04: Its chemical formula is $HO.C_6H_4.COONa$:



It is light-sensitive and must, therefore, be kept in an opaque container.

Sodium acetate exists as colorless, transparent crystals or as granular powder. The molecular weight is 136.07 and it has a solubility of one gram in 0.8 ml. of water. It should be protected from conditions of high humidity and, as a precaution, it should also be stored in a dessicator. Its chemical formula is $NaC_2H_3O_2.3H_2O$. Reagent grade was used in these investigations.

Cultivation of the Cells

In all cases, cells were cultured in pyrex flasks that were sealed with silicone stoppers. Stock cultures were maintained in pyrex T-60 flasks, while cells used in experimental procedures were carried in T-15 flasks, Leighton tubes, or Carell flasks.

All flasks were prepared for use by soaking in a 1:100 solution of Cytoclean (Isolab, Inc., Akron, Ohio) for a period of 24 hours. They were then rinsed and placed in dichromic acid for a similar period after which they were rinsed fifteen times in tap water and three times in distilled water. Coverslips or slides used in Leighton tubes were scrubbed gently in household cleanser, rinsed, ultrasonically cleaned in a solution of Cytoclean and then rinsed in tap water 15 times and distilled water three times. All other glassware used in these investigations was soaked for 24 hours in Cytoclean and rinsed as above. After final rinsing in distilled water, all glassware was dried, wrapped, autoclaved (25 minutes at 250°F), and then placed in a drying oven at 150°F for several hours before use.

The medium used was NCTC 109 plus 10% newborn calf serum. This medium is available in powdered form and was obtained from Grand Island Biological Company, Berkeley, Ca. The serum which was added during preparation of the medium, obtained from North American Biological, Gardena, Ca. The contents of the medium are listed in Table I.

Sterilization of the medium was accomplished by filtration through a Millipore filter with a pore size of 0.22 micron. The medium was then stored in 600 ml. serum bottles at 4°C. until needed. At the time of use, it was warmed to approximately room temperature and refiltered through a 0.22 micron Millipore.

The medium was changed three times per week in the stock cultures. This was accomplished by drawing off the old medium in the flasks with a Pasteur pipette attached to a vacuum evacuator and adding a minimum of 12 ml. of fresh medium from a dispensing flask. Each culture was gassed with a mixture of 95% air and 5% CO_2.

When subculturing these stocks, the cells were harvested with the aid of a nichrome scraper and the resulting suspension was then aspirated into a 10 ml. pipette and divided equally between four new flasks. Additional fresh medium was added, the cultures were gassed, sealed and then placed in the incubator at 37°C.

Experimental Procedure

All cultures, other than stocks, were replicated according to the method of Pace and Aftonomos (1957). During the course of this study a further modification of the replicator was developed. While this modification does not alter the basic design of the original device, hence retaining its reliability, it does make it possible to add the gas phase (95% air and 5% CO_2) as the cell suspension is introduced into the culture flask. Previously, this was done after the introduction of the cell suspension by using a separate gassing device. By reducing the number of manipulations involved, this system gives greater gassing consistency, lessens the chance for contamination, and saves a considerable amount of time.

The modification involves only the dispensing bell of the replicator. The original type is shown in Figure 1a and the modification is shown in figure 1b.

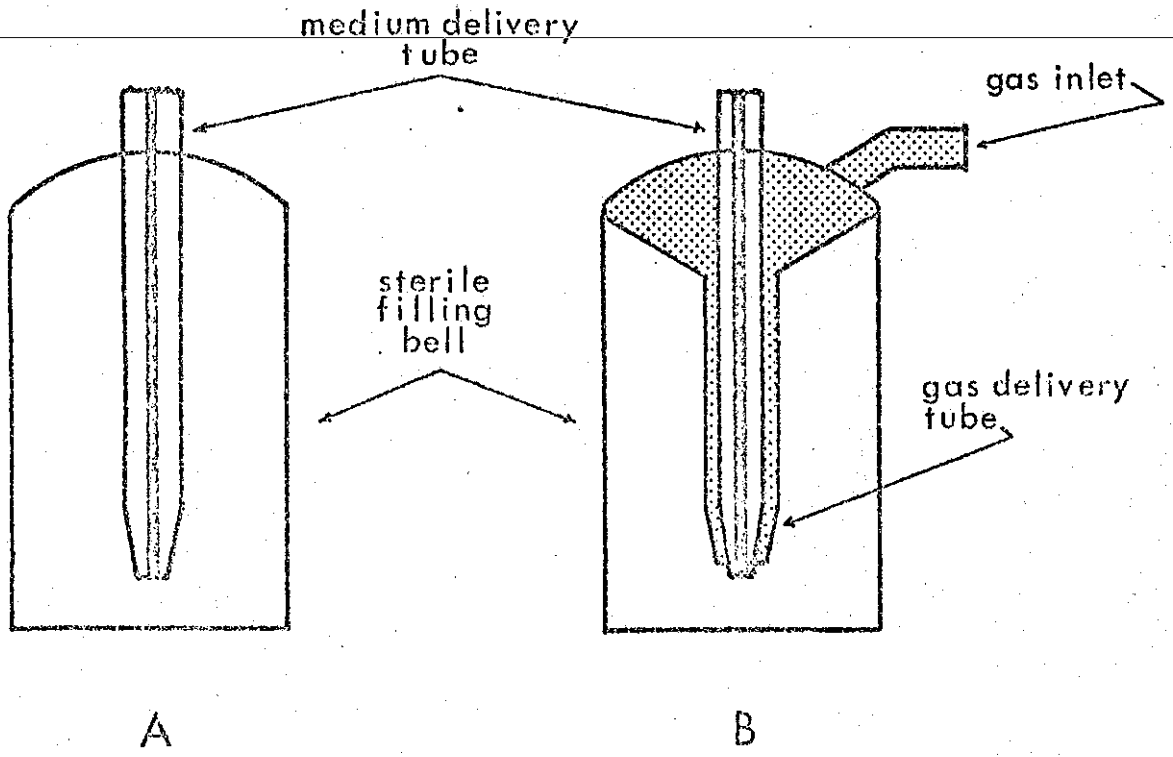
The cells to be replicated were harvested during the log phase by the same method used for subculturing. In order to obtain an

FIGURE 1. Modification to the replicating device.

A. The unmodified sterile filling bell

B. The sterile filling bell modified to include the gassing mechanism .

FIGURE 1


medium delivery tube
gas inlet
sterile filling bell
gas delivery tube
A
B

innoculum of approximately 100,000 cells per ml., the cells in one half of the number of stock cultures thought to be necessary for the experiment were added to the replicator and to this was added one-half the amount of medium required to set up the run. A cell count was then made with the aid of a Coulter Counter (model B), the necessary adjustments were made, and then the remainder of the medium was added. The cells were maintained in a uniform suspension by means of a magnetic stirrer (Pace & Aftonomos, 1957). After ten minutes, or longer, the suspension was sufficiently uniform to proceed with the replication at which time cell counts were made.

Replicate cultures were prepared by adding 2 ml. of the cell suspension to the T-15's and 1.5 ml. to the Leighton tubes and Carell flasks. The cultures were then gassed, sealed with silicone stoppers and placed in the incubator. For studies on growth, glucose utilization and lactic acid production, 70 T-15 flasks were prepared; four were used for preliminary counts and the others were divided into four experimental groups and one control group. Ten to fifteen Leighton tubes were used for fat determinations and ten Carell flasks, for each time-lapse study. Following replication, the cells were allowed 48 hours to attach and adjust before being subjected to experimental conditions.

At 48 hours, solutions of the compounds under investigation were prepared by placing the appropriate amount of each substance in an Erlenmeyer flask with amount of medium required and then spinning each with the aid of a magnetic stirrer for 30 minutes. In all cases, solutions were prepared immediately before use.

Resterilization and addition of the medium was accomplished in

one step with the aid of a Millipore filter affixed to a 30 ml. syringe. Three such syringes were necessary. One for the control cultures which, after use, was flushed with the aspirin solution and then used for the cultures to which the aspirin was to be added. The second was used to add the sodium salicylate and then flushed with the medium containing sodium salicylate and sodium acetate and used to add that combination. The third was used for the sodium acetate solution. In all cases, the proper amount was added to each flask after the complete removal of the old medium by means of the evacuator and Pasteur pipettes. After fresh medium was added to each, it was gassed and resealed.

Growth Measurement

In this study, increase in cell number was taken as an indication of "growth". Twenty-four hours after each medium change, two T-15 flasks in each group were removed and the cell number ascertained with the aid of the Coulter Counter. Before addition of the counting solution, the medium was removed and the two samples in each category were pooled and frozen for later analysis.

Chemical Analysis of the Media

Twenty-four hour samples of the different 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 reproducible results, and has good sensitivity and specificity while retaining the simplicity of the original method. Absorbance at 630 mμ was measured by means of a Coleman Hitachi 101 or Hitachi Perkin-Elmer 139 Spectrophotometer.

Lactate was determined by the method of Marbach and Weil (1967). This technique is essentially the same as that used in previous enzymatic methods with the exception of the precipitant. In this instance metaphosphoric acid is used because it does not affect the stability of NADH in the reaction mixture.

The reaction is as follows:

$$\text{l-lactate} + NAD^{+} \xrightarrow{LDH} \text{pyruvate} + NADH + H^{+}$$

Although equilibrium favors the formation of lactate and NAD^{+}, the reaction can be shifted completely in favor of pyruvate and NADH by using hydrazine to trap the formed pyruvate, an alkaline medium, and an excess of NAD^{+}. The amount of NADH produced was ascertained with the aid of a Beckman ACTA C II spectrophotometer (340 mμ).

Cytochemical Determinations

Fat content of the cells was ascertained by means of the stain, Oil-red-O after they were subjected to experimental conditions for a period of five days. The stain was added while the coverslips were still within the Leighton tubes.

The medium was first removed and the cells fixed with neutral buffered formalin. They were then washed with double distilled water and prepared to accept the stain by immersing them in 100% propylene glycol for five minutes. After this, they were stained with Oil-Red-O (in propylene glycol) for seven minutes. Differentiation was achieved by removing the stain and covering the cells with an aqueous solution containing 85% propylene glycol (volume for volume). This was followed by a wash in double distilled water and the coverslips were then removed from the Leighton tubes and mounted on standard size slides using glycerogel as the mounting medium. Fat deposits appear pink

to red in color while the rest of the cell is essentially colorless.

Photomicrographic Techniques

Cells to be photographed in the living state were cultured in T-15 or thin bottomed Carrel flasks, while those to be fixed and stained were cultured on number one coverslips in Leighton tubes.

While the optical properties of the T-flasks were relatively good, they are not suitable when extremely high quality photomicrographs are desired; however, for general purposes, they are adequate and their use makes it unnecessary to set up extra cultures specifically for photography.

For time-lapse studies, the thin-bottom Carrel flasks proved to be well suited. In addition to being optically superior to the T-flasks, the medium within could be easily changed while the flask was affixed to the microscope stage.

Cells to be fixed and stained were cultured on number one coverslips in Leighton tubes. Because the coverslips are extremely thin and are optically flat, they are well suited for high resolution microscopy.

For color photography, a Zeiss model RA microscope fitted with Nomarski differential interference optics and a 35mm Zeiss camera was utilized. The film used was Kodak EHB 135.

For black and white photography, either still or time-lapse, an inverted model M Nikon with phase contrast optics was used. Still pictures were taken with a 35mm Nikon F equipped with a Nikon photomicrographic adaptor. The film used was Kodak Plus X Pan.

For time-lapse work, a Sage cinematographic unit was used. Direct positives were made with 16mm Kodak Plus X Reversal film

while negatives were made with Kodak Plus X. The correct temperature for the culture being studied was maintained by a Sage Air Curtain Incubator.

RESULTS

RESULTS

Growth Effects

Throughout the text of this report abbreviation or symbols will be used to represent the various compounds; for example, aspirin (ASA), sodium salicylate (Sal), sodium acetate (Ac), sodium salicylate and acetate (Sal-Ac), and control (Con).

Concentrations of 0.55 mM, 1.67 mM, and 2.78 mM were used for each compound or combination and a minimum of two experiments were made for each condition.

Table II shows the data collected during the experiments using a concentration of 0.55 mM, and Figures 2A and 2B present this material graphically. While it appears that there might have been some degree of growth inhibition among the experimental cultures by day three, the day five results do not substantiate this. At this time, the cells cultured in a medium containing ASA or Sal had populations that fell within the range of the control cultures in both experiments; cells exposed to Sal-Ac were moderately lower in number in one experiment and slightly higher in the other. In the case of Ac, the cell number was within the control range in the second run, but slightly higher in the first. At this concentration, then, little or no effect upon growth could be detected.

At 1.67 mM, the next higher concentration of the various compounds used, some effects on growth began to appear. The data obtained during the experiments is presented in Table III, with graphical representations in Figures 3A and 3B.

TABLE II

The effects of the various compounds under investigation, at a concentration of 0.55 mM, upon growth, glucose utilization, and lactate production in Low Line cells.

EXPERIMENT I

Time of exposure in days	Experimental condition	No. of cells per ml. ($x10^5$) Two cultures	Average no. of cells per ml.($x10^5$)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	1.10; 1.53	1.32		
1	Con	3.12; 3.30	3.21	7.06	6.58
	ASA	2.92; 3.58	3.25	7.45	6.86
	Sal	3.73; 3.58	3.79	8.36	6.32
	Sal-Ac	3.22; 3.40	3.31	9.28	8.47
	Ac	3.40; 3.85	3.62	8.25	6.55
3	Con	6.22; 8.00	7.11	4.79	6.53
	ASA	6.04; 6.48	6.26	6.06	6.96
	Sal	5.25; 7.35	6.35	5.48	5.78
	Sal-Ac	4.25; 7.22	5.74	6.45	8.52
	Ac	6.30; 7.80	7.05	6.71	7.76
5	Con	12.00; 12.30	12.20	3.29	3.21
	ASA	12.00; 12.40	12.20	3.31	3.16
	Sal	12.30; 12.30	12.30	3.62	4.32
	Sal-Ac	10.80; 11.00	10.90	3.79	4.65
	Ac	12.50; 12.60	12.60	3.56	4.57

EXPERIMENT II

Time of Exposure in days	Experimental condition	No. of cells per ml. ($x10^5$) Two cultures	Average no. of cells per ml. ($x10^5$)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	--------	-------		
1	Con	2.65; 3.48	3.06	11.30	17.80
	ASA	2.80; 3.08	2.94	6.22	16.20
	Sal	2.13; 2.65	2.40	3.78	19.30
	Sal-Ac	3.30; 3.45	3.38	2.11	13.60
	Ac	2.68; 2.90	2.79	2.80	12.70
3	Con	6.12; 6.62	6.37	5.61	14.60
	ASA	5.03; 5.32	5.18	5.37	14.10
	Sal	5.48; 5.55	5.52	6.17	20.60
	Sal-Ac	5.85; 6.00	5.92	4.60	16.60
	Ac	5.68; 6.05	5.86	4.04	13.20
5	Con	7.32; 7.80	7.56	3.44	19.60
	ASA	6.90; 7.38	7.14	4.84	12.80
	Sal	6.88; 7.58	7.23	5.47	17.30
	Sal-Ac	8.05; 8.10	8.08	4.63	12.50
	Ac	7.32; 7.40	7.36	5.67	17.80

FIGURE 2. The effects of the various compounds at a concentration of 0.55 mM on growth in Low Line cells. Each bar represents the average of two counts (indicated by vertical lines).

A. Experiment I

B. Experiment II

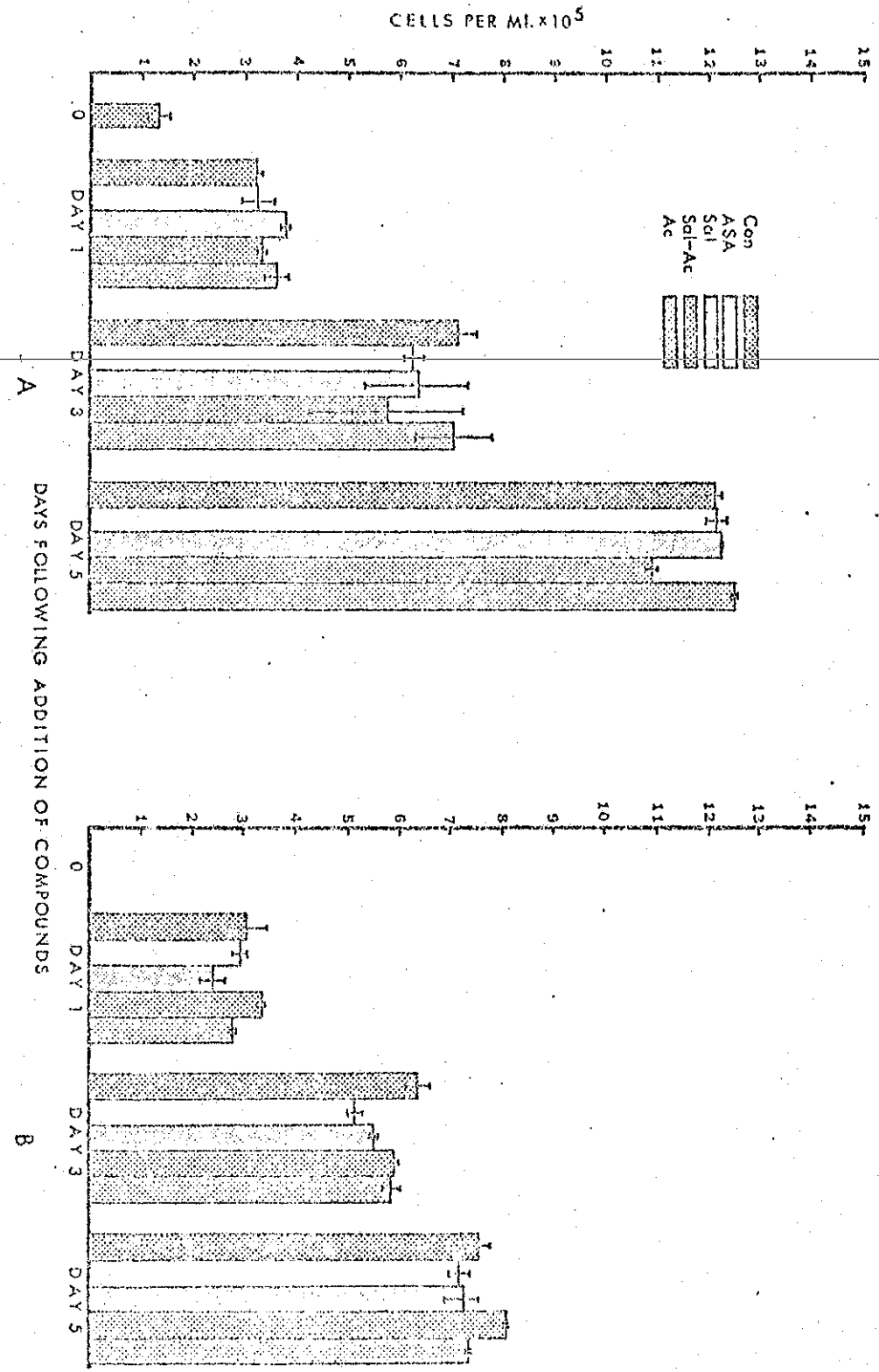

CELLS PER ML. x 10^5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0
DAY 1
DAY 3
DAY 5
Con
ASA
Sal
Sal-Ac
Ac
A
B
DAYS FOLLOWING ADDITION OF COMPOUNDS

FIGURE 2

TABLE III

The effects of the various compounds under investigation, at a concentration of 1.67mM., upon growth, glucose utilization, and lactate production in Low Line cells.

EXPERIMENT I

Time of exposure in days	Experimental condition	No. of cells per ml.($x10^5$) Two cultures	Average No. of cells per ml.($x10^5$)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	1.85; 2.50	2.18		
1	Con	4.88; 5.00	4.94	4.59	13.00
	ASA	4.78; 4.95	4.86	4.88	16.30
	Sal	4.92; 5.32	5.12	5.20	12.20
	Sal-Ac	4.62; 4.78	4.70	5.89	14.00
	Ac	4.60; 4.62	4.61	6.67	18.10
3	Con	8.08; 8.98	8.53	2.92	4.80
	ASA	8.15; 8.80	8.48	3.34	9.83
	Sal	8.10; 8.82	8.46	3.96	7.43
	Sal-Ac	8.20; 8.98	8.59	3.77	6.51
	Ac	8.50; 8.95	8.72	3.84	8.35
5	Con	11.70; 11.90	11.80	2.55	3.47
	ASA	11.30; 11.30	11.30	4.42	7.00
	Sal	11.00; 11.30	11.20	3.23	7.30
	Sal-Ac	10.60; 11.20	10.90	3.38	6.68
	Ac	12.90; 13.60	13.20	2.28	3.03

EXPERIMENT II

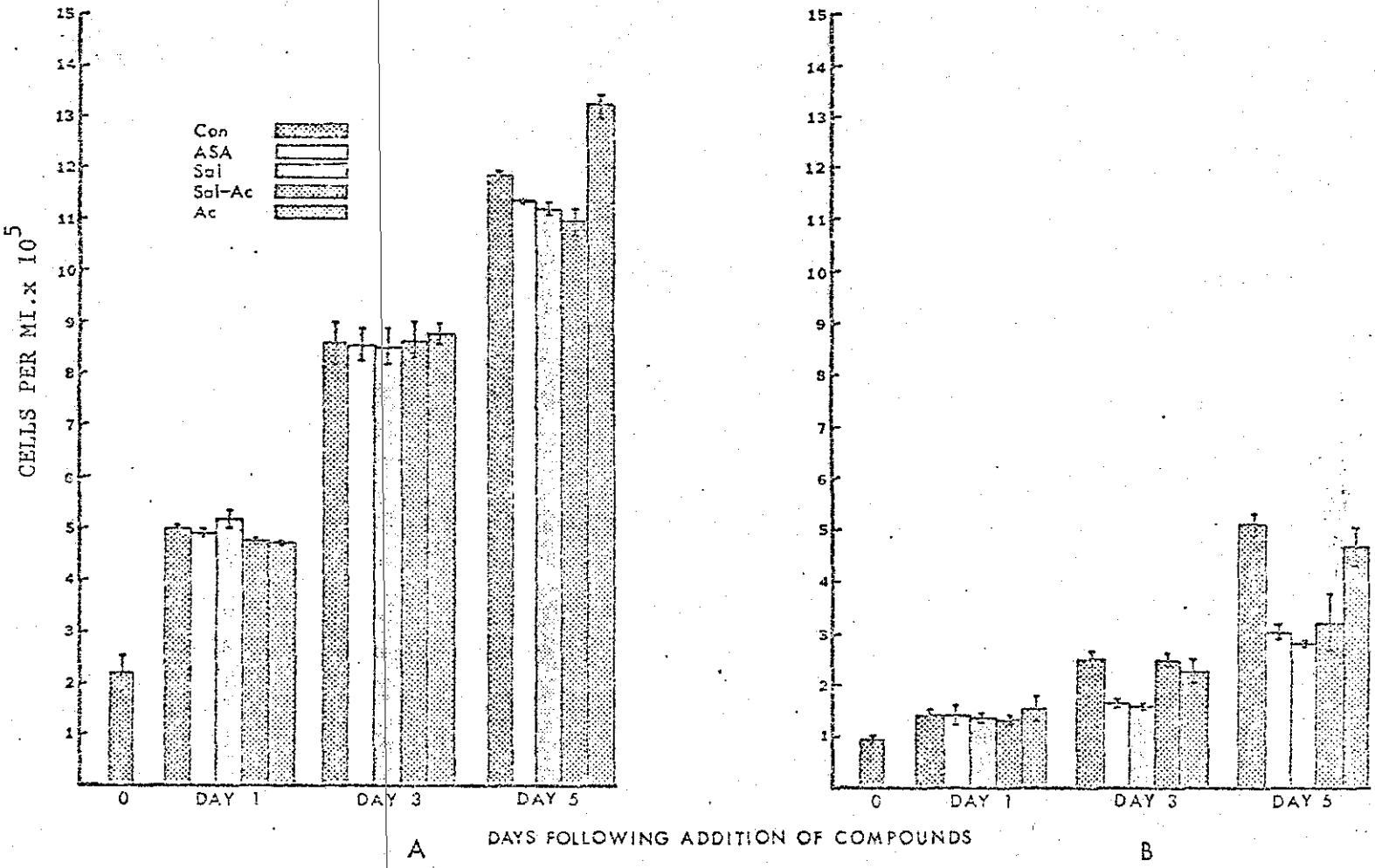
Time of exposure in days	Experimental condition	No. of cells per ml.($x10^5$) Two cultures	Average no. of cells per ml.(10^5)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	0.832; 0.932			
1	Con	1.22; 1.45	1.34	12.30	42.20
	ASA	1.17; 1.52	1.34	14.10	40.40
	Sal	1.22; 1.42	1.32	14.80	46.70
	Sal-Ac	1.18; 1.37	1.28	14.80	39.00
	Ac	1.27; 1.72	1.50	13.80	33.90
3	Con	2.32; 2.62	2.47	11.70	33.20
	ASA	1.51; 1.70	1.60	18.10	43.80
	Sal	1.46; 1.58	1.62	17.20	75.00
	Sal-Ac	2.30; 2.55	2.43	11.70	34.40
	Ac	1.95; 2.46	2.20	13.40	43.30
5	Con	4.85; 5.30	5.08	8.37	26.40
	ASA	2.78; 3.12	2.95	12.80	41.30
	Sal	2.72; 2.80	2.76	13.90	43.60
	Sal-Ac	2.60; 3.72	3.16	12.20	37.00
	Ac	4.22; 4.98	4.60	9.89	32.20

FIGURE 3. The effects of the various compounds at a concentration of 1.67 mM on growth in Low Line cells. Each bar represents the average of two counts (indicated by vertical lines).

A. Experiment I

B. Experiment II

FIGURE 3


CELLS PER ML. x 10^5
Con
ASA
Sal
Sal-Ac
Ac
0
DAY 1
DAY 3
DAY 5
A
B
DAYS FOLLOWING ADDITION OF COMPOUNDS

While it is obvious that proliferation got off to a considerably slower start in the second experiment at this concentration, and that the compounds were therefore added at a time when fewer cells were present, the end results of both experiments were similar in most respects.

In the first experiment at 1.67 mM (Figure 3A), the compounds appeared to exert no effect until the fifth day. At that time, a slight inhibition of growth in the cultures exposed to ASA, Sal, and Sal-Ac could be seen. The cells exposed to Ac alone were moderately higher in number than those of the control cultures.

In the second run, some effect could be seen by day 3 (Figure 3B). In cultures exposed to ASA and Sal, a moderate inhibition was evident while little or no effect could be seen in the case of Sal-Ac or the Ac alone; however, by the fifth day there was marked inhibition in the cultures exposed to Sal-Ac as well as those exposed to ASA and Sal. The cells exposed to Ac alone increased considerably in number by that time and, while there was some overlapping of range, their average number was higher than that of the controls.

It appears then, at a concentration of 1.67 mM, ASA, Sal, and Sal-Ac are somewhat inhibitory to growth. This effect is more pronounced when the compounds are added to cultures that are initially lower in number and/or have had a slower growth rate in general. Ac gives evidence of being stimulatory to growth in conditions of high cell density while having little or no effect under less crowded conditions.

Data collected during the growth studies at the highest concentration (2.78 mM) are presented in Table IV and Figures 4A and 4B.

TABLE IV

The effects of the various compounds under investigation, at a concentration of 2.78 mM., upon growth, glucose utilization, and lactate production in Low Line cells.

EXPERIMENT I

Time of exposure in days	Experimental condition	No. of cells per ml.($x10^5$) Two cultures	Average no. of cells per ml.($x10^5$)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	2.60; 2.90	2.75		
1	Con	5.58; 6.12	5.85	4.24	16.30
	ASA	5.22; 5.40	5.31	5.33	19.70
	Sal	4.98; 5.40	5.19	6.11	23.10
	Sal-Ac	5.55; 5.78	5.66	5.61	20.70
	Ac	6.48; 6.50	6.49	5.12	18.60
3	Con	9.90; 10.30	10.10	3.52	10.20
	ASA	6.00; 6.15	6.05	6.83	24.00
	Sal	7.50; 8.55	8.02	5.14	17.00
	Sal-Ac	8.65; 9.00	8.82	4.88	14.30
	Ac	10.20; 10.40	10.30	3.54	9.98
5	Con	11.90; 13.00	12.40	2.79	7.43
	ASA	6.90; 7.05	6.98	5.83	21.60
	Sal	9.52; 10.10	9.81	4.34	14.20
	Sal-Ac	10.80; 11.10	11.00	3.99	13.30
	Ac	14.00; 14.40	14.20	2.90	7.28

EXPERIMENT II

Time of exposure in days	Experimental condition	No. of cells per ml.($x10^5$) Two cultures	Average no. of cells per ml.($x10^5$)	Glucose utilized ($ugx10^{-3}$)	Lactate produced ($ugx10^{-3}$)
0	Con	1.88; 2.36	2.12		
1	Con	4.50; 5.18	4.84	4.50	15.10
	ASA	4.72; 4.70	4.56	4.67	15.70
	Sal	4.62; 4.87	4.75	4.38	16.30
	Sal-Ac	3.65; 4.25	3.95	6.04	20.60
	Ac	5.15; 5.35	5.25	5.77	17.80
3	Con	7.82; 8.08	7.95	4.47	13.20
	ASA	7.02; 7.30	7.16	6.00	19.80
	Sal	7.80; 8.42	8.11	5.08	16.40
	Sal-Ac	8.67; 8.70	8.69	4.63	14.00
	Ac	9.52; 9.72	9.62	3.50	10.40
5	Con	10.80; 12.50	11.60	2.70	5.86
	ASA	8.27; 9.25	8.76	4.86	16.20
	Sal	8.80; 9.52	9.16	4.60	13.70
	Sal-Ac	10.90; 11.00	11.00	3.76	12.00
	Ac	11.80; 12.40	12.10	3.33	8.75

FIGURE 4. The effects of the various compounds at a concentration of 2.78 mM on growth in Low Line cells. Each bar represents the average of two counts (indicated by vertical lines).

A. Experiment I

B. Experiment II

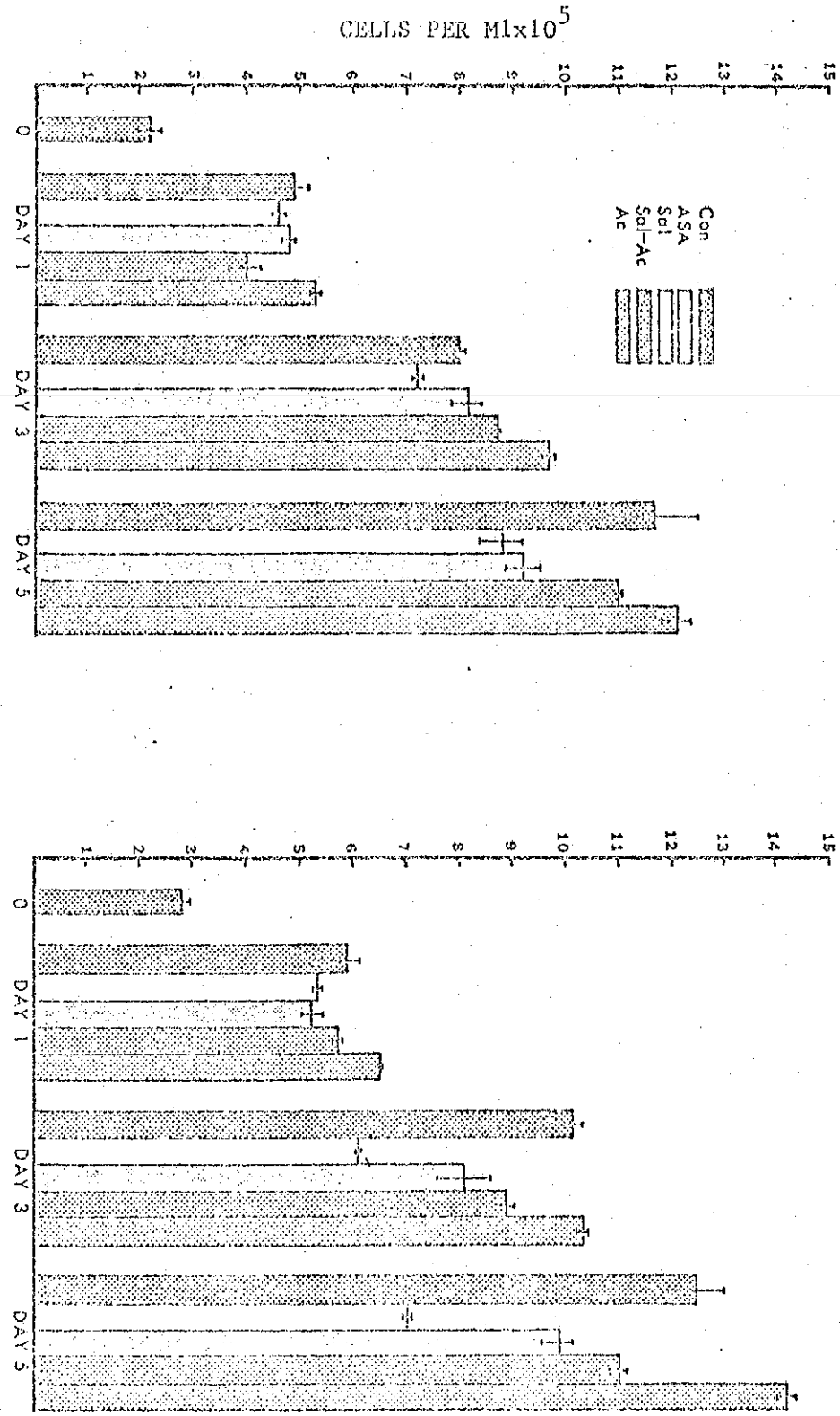

CELLS PER Mlx10^5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0
DAY 1
DAY 3
DAY 5
Con
ASA
Sal
Sal-Ac
Ac
A
DAYS FOLLOWING ADDITION OF COMPOUNDS
B

FIGURE 4

Here, a relatively clear trend appeared by day 3. The greatest inhibitory effect was exerted by ASA, followed by Sal and then Sal-Ac. While cell number in the cultures exposed to Sal was within the range of the controls in the first run (Figure 4A) results at day 5 of that particular experiment plus day 3 and day 5 of the second run (Figure 4B) seem to indicate that this count may not have been representative. Sodium acetate had a stimulatory effect that appeared by day 3 in both experiments.

Morphologically, cells exposed to the various compounds at this concentration for a period of five days were different in one significant respect from the controls. In each case, while the cell body did not show any noticeable change, the number and character of the protoplasmic extensions was altered. In all instances, there was an increase in their number as well as in the degree of bifurcation; the amount of material present in these extensions was also greater, possibly indicating increased pinocytotic activity. Photomicrographs taken at this time are shown in Figure 5.

Glucose Utilization and Lactate Production

During the course of the growth studies, glucose utilization and lactate production were ascertained. The results obtained for glucose uptake are given in Tables II, III, and IV and presented graphically in figures 6, 7, and 8 (0.55 mM, 1.67 mM, and 2.78 mM respectively). It will be noted that under control conditions there is a decrease in the amount of glucose utilized with time which is actually related to increase in cell number. While cells exposed to the various compounds under investigation generally produced patterns that were similar, the quantities utilized were usually greater.

At concentrations of 0.55 mM, the compounds had little if any consistent effect; however, by day 5, the amounts of glucose taken up appeared to be equal to or greater than in the controls. (Figure 6).

FIGURE 5. Photomicrographs showing the effects of exposure to the various compounds at a concentration of 2.78 mM for a period of five days.

A. Aspirin

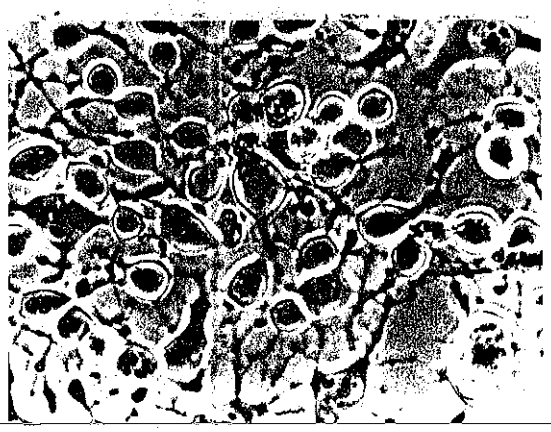
B. Sodium salicylate

C. Control

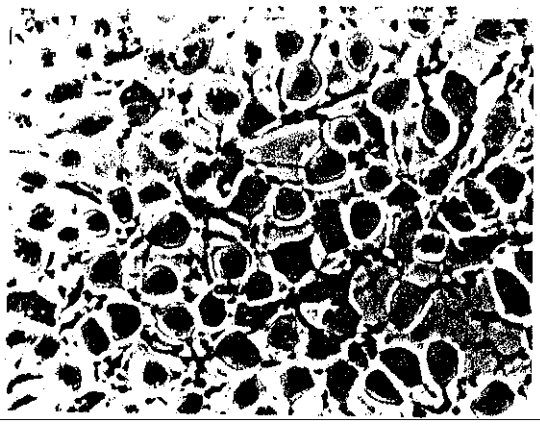
D. Sodium salicylate and sodium acetate

E. Sodium acetate

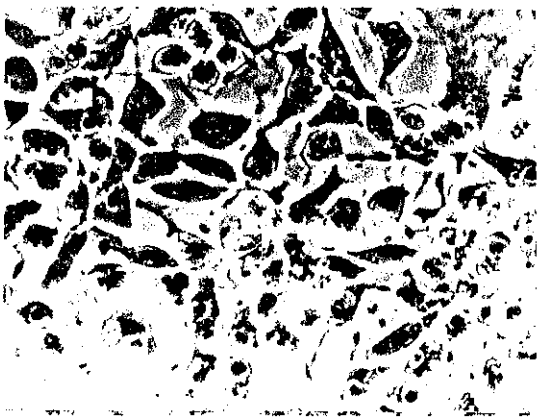
FIGURE 5



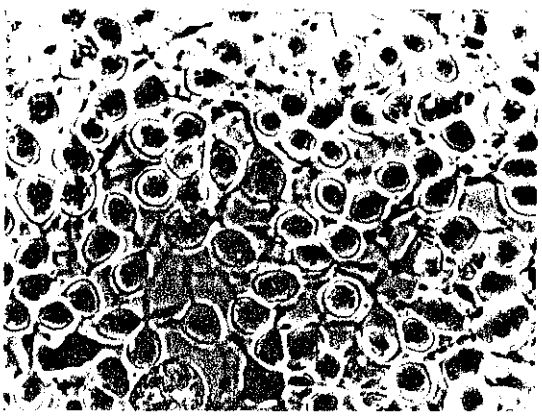
A



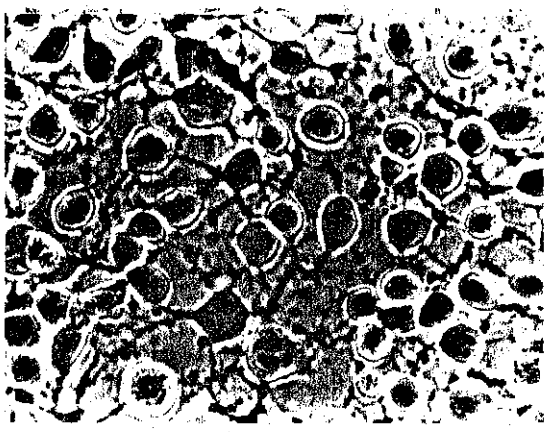
B



C



D



E

FIGURE 6. The effects of the various compounds at a concentration of 0.55 mM on glucose utilization in Low Line cells.

A. Experiment I

B. Experiment II

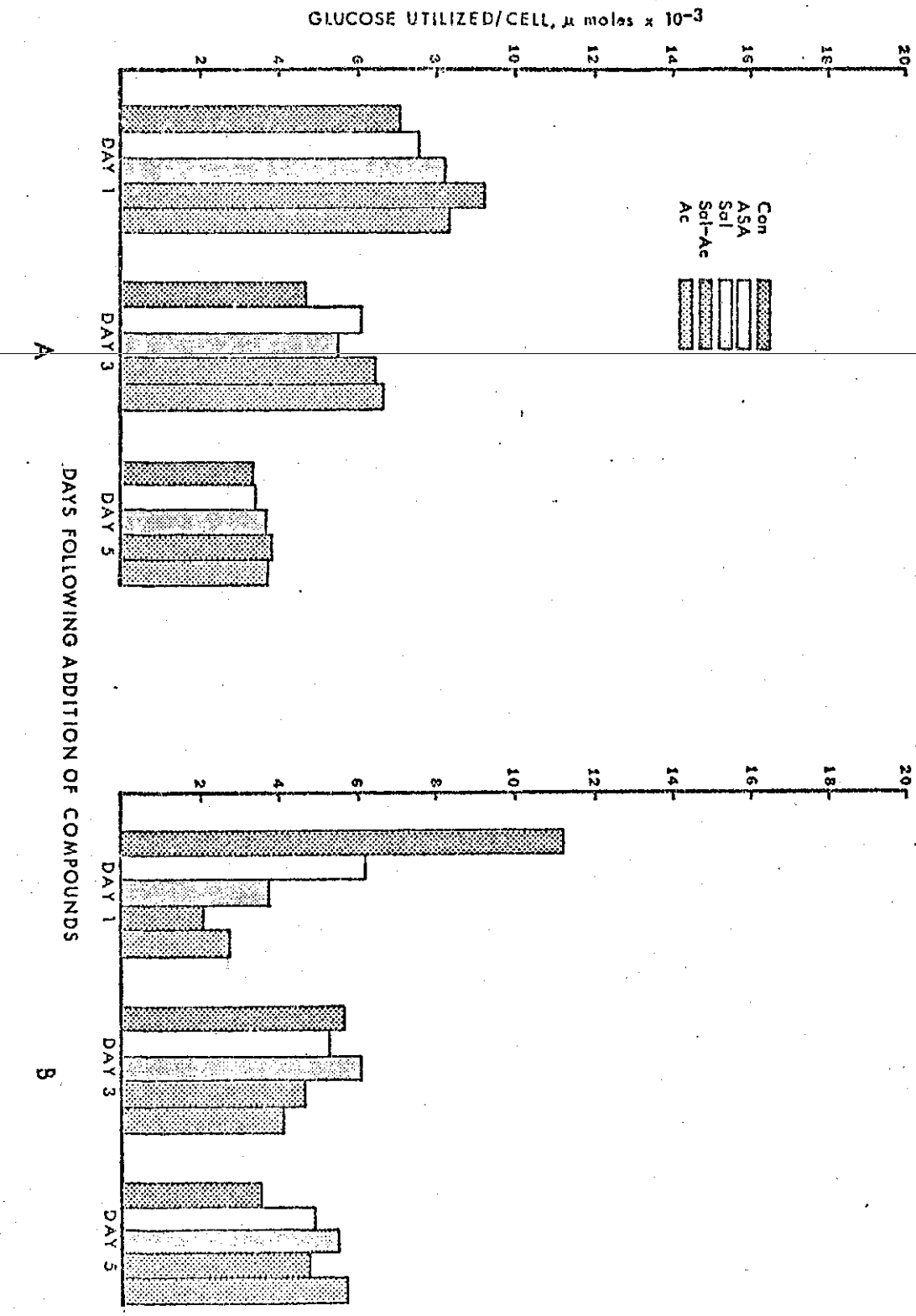

GLUCOSE UTILIZED/CELL, μ moles x 10⁻³
2
4
6
8
10
12
14
16
18
20
DAY 1
DAY 3
DAY 5
Con
ASA
Sal
Sal-Ac
Ac
A
B
DAYS FOLLOWING ADDITION OF COMPOUNDS

FIGURE 6

FIGURE 7. The effects of the various compounds at a concentration of 1.67 mM on glucose utilization in Low Line cells.

A. Experiment I

B. Experiment II

FIGURE 7

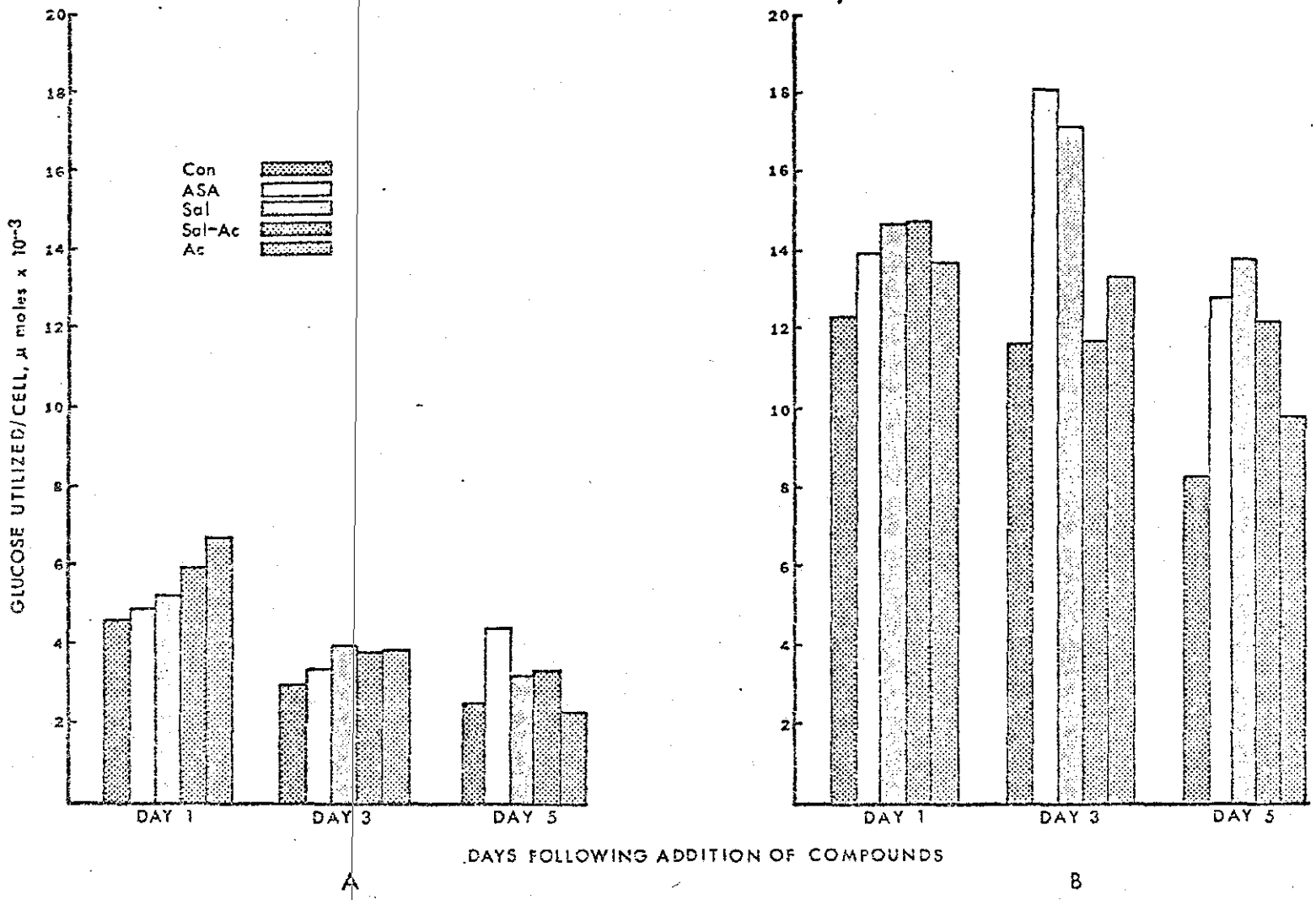
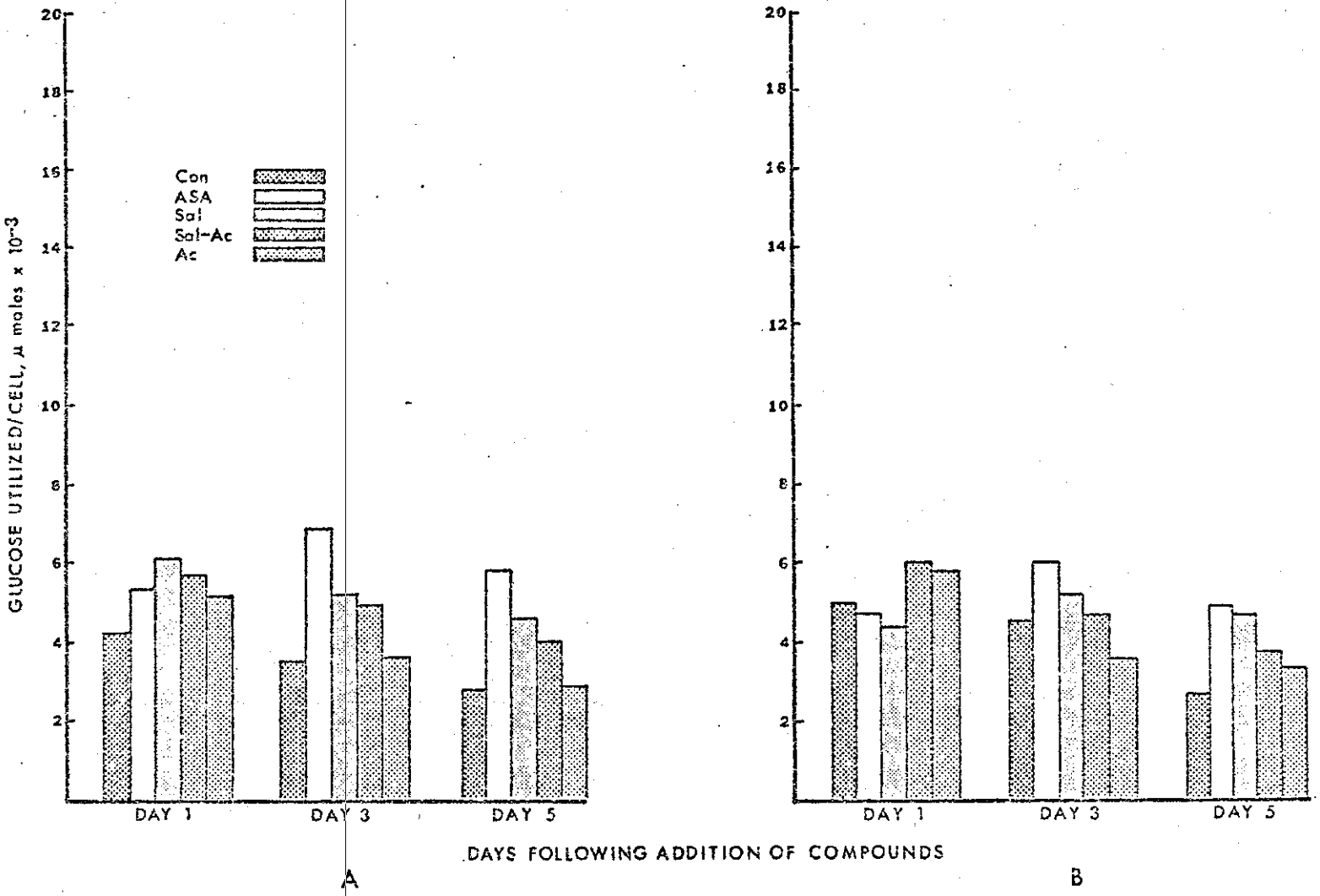

GLUCOSE UTILIZED/CELL, μ moles x 10⁻³
Con
ASA
Sal
Sal-Ac
Ac
DAY 1
DAY 3
DAY 5
DAYS FOLLOWING ADDITION OF COMPOUNDS
A
B

FIGURE 8. The effects of the various compounds at a concentration of 2.78 mM on glucose utilization in Low Line cells.

A. Experiment I

B. Experiment II

FIGURE 8


GLUCOSE UTILIZED/CELL, μ moles x 10⁻³
20
18
16
14
12
10
8
6
4
2
Con
ASA
Sal
Sal-Ac
Ac
DAY 1
DAY 3
DAY 5
A
B
DAYS FOLLOWING ADDITION OF COMPOUNDS

At 1.67 mM, there was considerable variation among the experimental groups, but, in almost every instance, glucose utilization per cell was greater than in the control cultures (Figure 7). In the second experiment at this concentration, uptake per cell was considerably higher than that encountered in any other. This was apparently related to an unusually low number of cells and a slower than normal growth rate.

At the higher concentration used (2.78 mM), a clear trend was established (Figure 8). ASA resulted in the greatest amount of glucose utilization (roughly twice that of the controls), followed, in order, by Sal, Sal-Ac, and finally Ac.

Closely related to glucose utilization is lactate production. The results of this portion of the study are shown in Tables II, III, and IV, with graphical representations in Figures 9, 10, and 11.

In general, the results varied directly with glucose uptake and, at a concentration of 2.78 mM ASA the greatest amount of lactate was produced (Figure 11). Exposure to other compounds at this concentration also resulted in a greater production of lactate in the following order: Sal Sal-Ac Ac. In the second experiment at this concentration Ac appeared to cause an increase by day 5. However, the results obtained for days 3 and 5 of the first experiment as well as the day 3 results of the second, indicate that the amount produced was either similar or less than that of the controls.

The relationship between lactate production and glucose utilization (at 2.78 mM) as demonstrated by the glycolytic ratio is revealed in Table V. The results obtained at day 3 were selected because this allowed enough time for the compounds to exert their

FIGURE 9. The effects of the various compounds at a concentration of 0.55 mM on lactic acid production in Low Line cells.

A. Experiment I

B. Experiment II

FIGURE 9

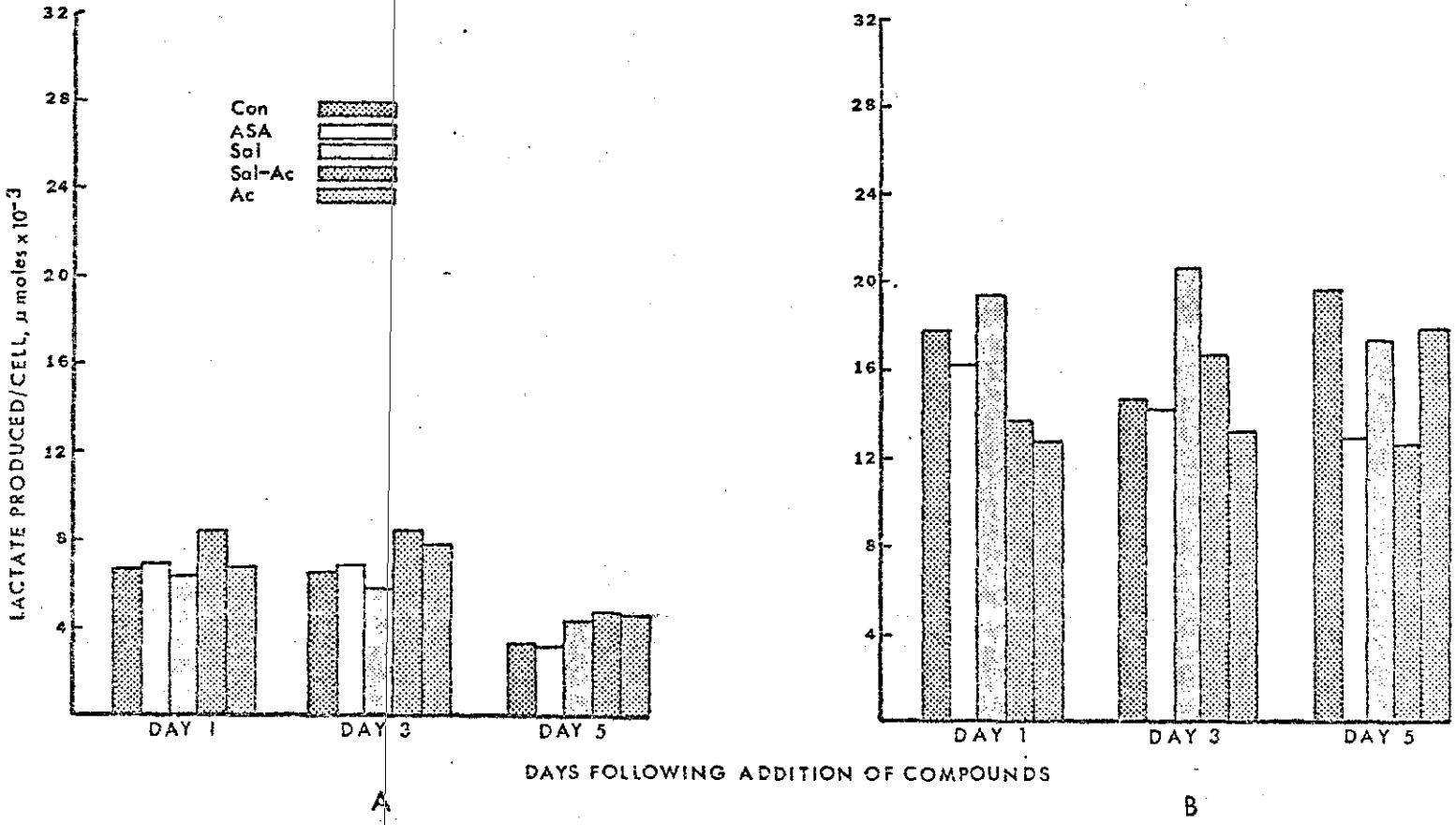

LACTATE PRODUCED/CELL, μ moles x 10⁻³
32
28
24
20
16
12
8
4
Con
ASA
Sal
Sal-Ac
Ac
DAY 1
DAY 3
DAY 5
A
B
DAYS FOLLOWING ADDITION OF COMPOUNDS

FIGURE 10. The effects of the various compounds at a concentration of 1.67 mM on lactic acid production in Low Line cells.

A. Experiment I

B. Experiment II

FIGURE 10

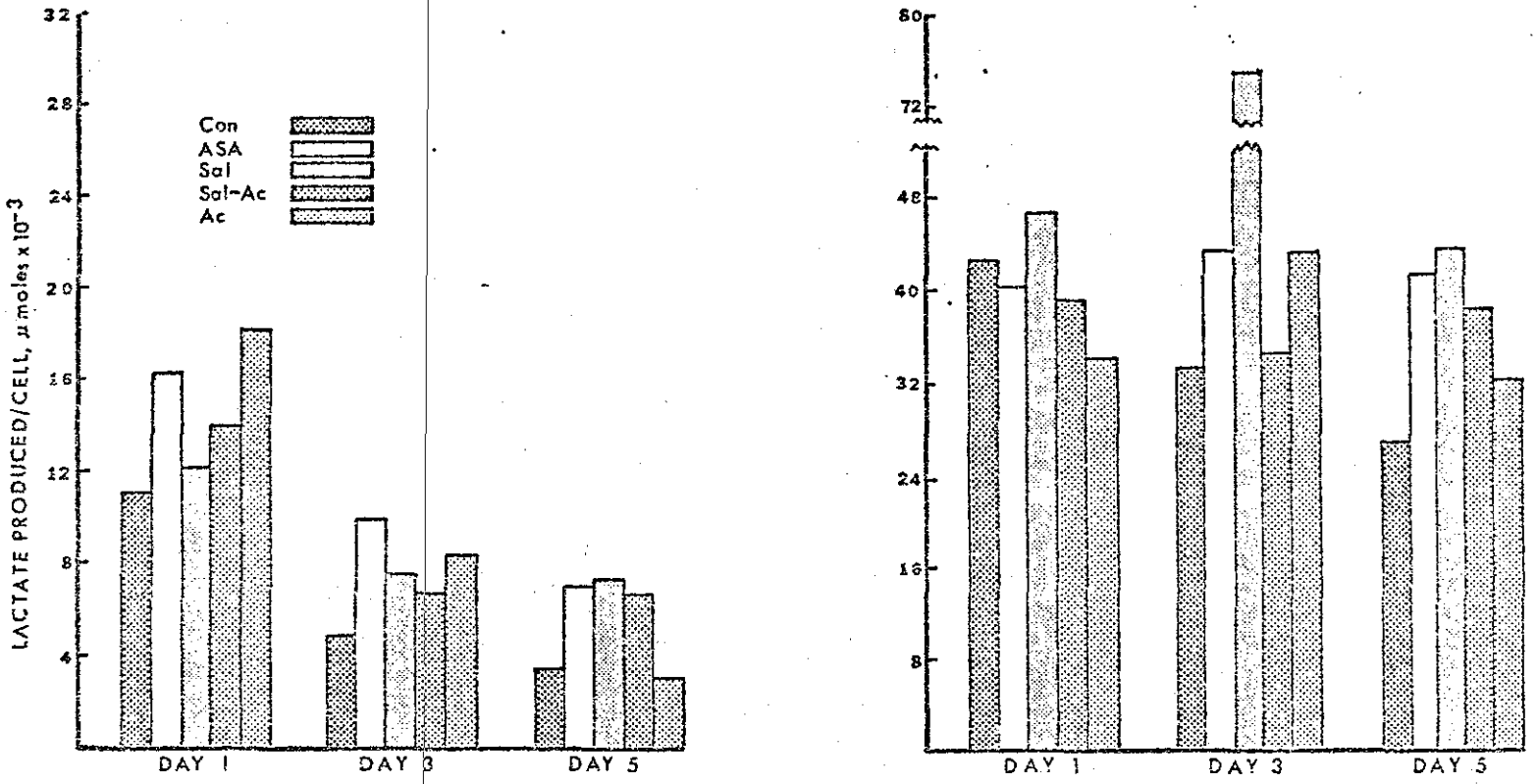

LACTATE PRODUCED/CELL, μ moles x 10⁻³
32
28
24
20
16
12
8
4
Con
ASA
Sal
Sal-Ac
Ac
DAY 1
DAY 3
DAY 5
80
72
48
40
32
24
16
8
DAY 1
DAY 3
DAY 5
DAYS FOLLOWING ADDITION OF COMPOUNDS
A
B

FIGURE 11. The effects of the various compounds at a concentration of 2.78 mM on lactic acid production in Low Line cells.

A. Experiment I

B. Experiment II

FIGURE 11

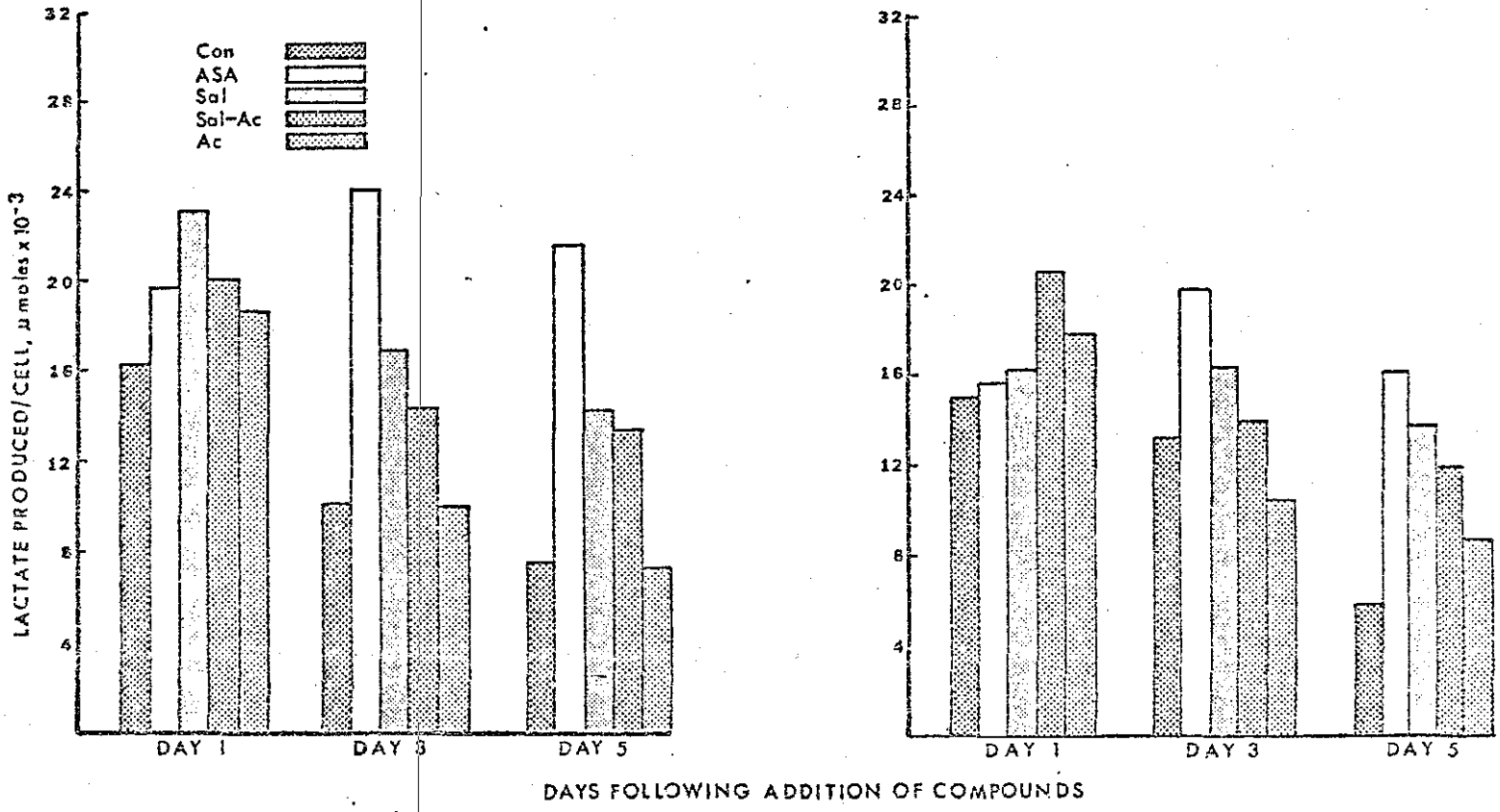

Con
ASA
Sal
Sal-Ac
Ac
LACTATE PRODUCED/CELL, μ moles x 10⁻³
32
28
24
20
16
12
8
4
DAY 1
DAY 3
DAY 5
DAYS FOLLOWING ADDITION OF COMPOUNDS
A
B

TABLE V

The ratio of lactic acid produced to glucose utilized on exposure to the various compounds at a concentration of 2.78 mM for a period of three days.

EXPERIMENT I		EXPERIMENT II	
Con	2.90	Con	2.96
ASA	3.52	ASA	3.30
Sal	3.20	Sal	3.23
Sal-Ac	2.93	Sal-Ac	3.02
Ac	2.82	Ac	2.97

effects but avoided possible influence due to cell crowding that would occur by day 5.

Cells exposed to ASA (2.78 mM) had the highest glycolytic ratio, followed by those exposed to Sal and Sal-Ac. In the case of Ac, the ratio was either similar or slightly lower then that of the control cultures.

Fat Determinations

The degree of intracellular fat deposition after exposure to experimental conditions for a period of three days was determined by staining with Oil-Red-O. While an attempt was made to photograph representative areas, it should be noted that even within the same culture there was variation in the number and size of the fat droplets present.

The photomicrographs in Figure 12 indicate that the least amount of fat was found in cultures exposed to the Sal-Ac combination. The control cells were next in order, with increasing amounts of fat appearing in cells exposed to ASA, Sal, and finally Ac. It is felt, however, that these results may not represent a true relationship between the compounds under study and the effects observed. Results of a second experiment which was continued for a period of six days after addition of the compounds tend to confirm this.

Time-lapse Studies

In order to gain a greater understanding of how these compounds might effect cells in a dynamic sense, time-lapse studies were made. Aspirin, apparently the most potent growth inhibitor of the group, was selected and used at a concentration of 3.3 mM. The filming rate

FIGURE 12. Photomicrographs showing the effects of the various compounds on fat deposition after exposure to a concentration of 2.78 mM for a period of three days.

A. Aspirin

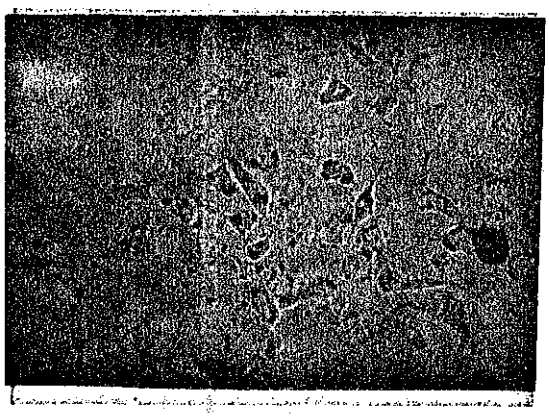
B. Sodium Salicylate

C. Control

D. Sodium salicylate and sodium acetate

E. Sodium acetate

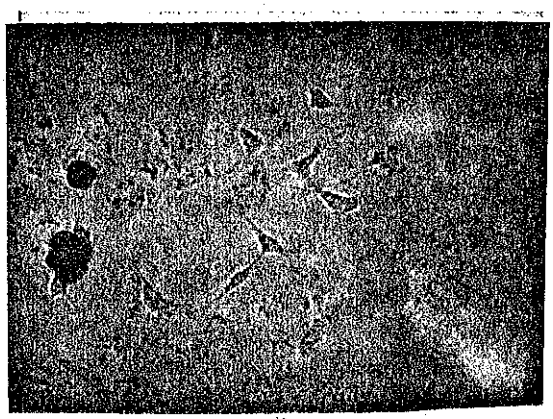
FIGURE 12



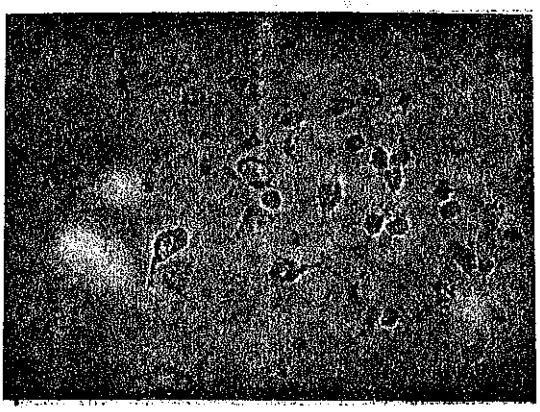
A

B

C



D



E

was one frame every 45 seconds and, after observing the cells for a period of eight hours under control conditions, the medium was removed and that containing the ASA was added. Observation was then continued for a period of 30 hours.

On exposure to ASA, several of the "giant" cells immediately rounded and within a few hours, some of these ceased all movement. During the last hours of this study, three of the remaining "giant" cells lysed in rapid succession. Cells of normal morphology appeared to be relatively unaffected; however, it is significant that no mitotic activity was noted during the 30 hour period.

Enlargements of selected frames exposed during this sequence are shown in Figure 13.

FIGURE 13. Selected frames from a time-lapse study of the effects of aspirin at a concentration of 3.3 mM.

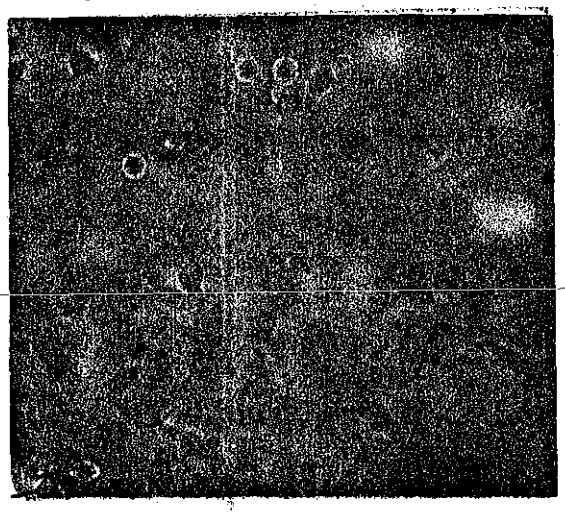
A. A frame taken immediately before the medium was exchanged for that containing the aspirin.

B. A frame taken immediately after addition of the medium containing aspirin. Several rounded cells can be seen.

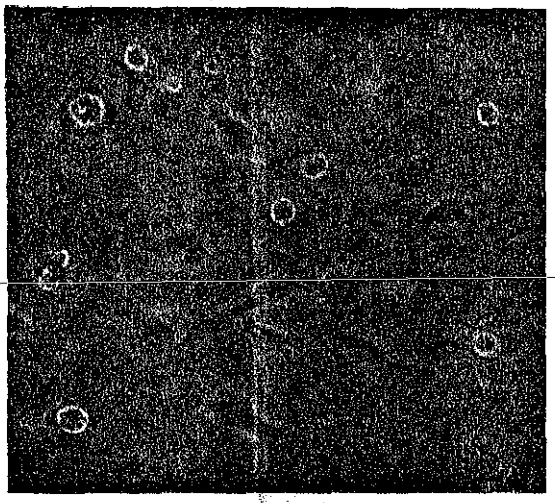
C. At approximately 25 hours after addition of the aspirin a "giant" cell lysed (arrow).

D. At approximately 30 hours two more "giant" cells lysed (arrows).

FIGURE 13



A



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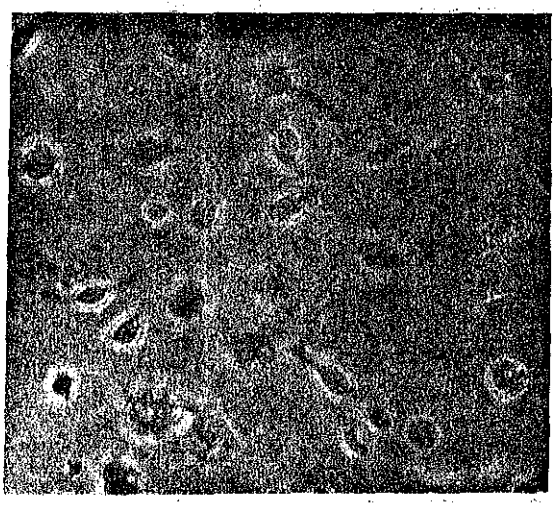

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D I S C U S S I O N

DISCUSSION

The first step in the biotransformation of aspirin is hydrolysis. It has been shown by Leonards (1962) to occur at a rapid rate in dog, the $t_{\frac{1}{2}}$ value being on the order of 0.30 hour. This means that if it has unique effects they must be initiated in a relatively short period of time or the breakdown products, salicylate and acetate, may act together in a manner different from salicylate alone. The combined effect of acetylsalicylate, salicylate, and acetate is, of course, the remaining possibility.

In this study, the effects of aspirin were compared to those of sodium salicylate, a combination of sodium salicylate and sodium acetate, and sodium acetate alone in an effort to obtain a greater amount of information relating ot the subject. Concentrations of 0.55 mM, 1.67 mM, and 2.78 mM were used for all but the time-lapse studies. In terms of plasma levels, these would cover the therapeutic to toxic range in man. The lower concentration would be encountered when only analgesia is desired, while the intermediate would be attained in the management of inflammation and the highest could prove fatal.

The selection of these concentrations was based on the results of previous work by the author (1970) concerning the effects of aspirin on Low Line cells; during that investigation it was also shown that there is, at least, some variation in the degree of response for different cell types.

In this study it was found that, of the various compounds

considered, at a concentration of 2.78 mM, aspirin was most inhibitory to growth. Sodium salicylate and the combination of sodium salicylate and sodium acetate were less inhibitory. Sodium acetate alone appeared to be somewhat stimulatory. When one considers the fact that the salicylates are capable of uncoupling oxidative phosphorylation, stimulating ATP-ase activity, and inhibiting many dehydrogenases and transaminases, it is not surprising that they should have a negative effect on cellular proliferation. However, still unexplained is the reason why aspirin is more potent in this respect and why it is more potent that other salicylates in the management of pain, inflammation, and fever. Some authors, such as Martin (1963), propose that aspirin has a greater ability to reach the site of action than does salicylate and, once there, salicylate is released and the desired effect is accomplished. But others disagree. Smith and Smith (1966), for example, feel that aspirin has a unique action and propose that in the case of analgesia it has a greater ability to occupy the pores of surface of chemoreceptors for pain. No direct evidence has been given to support this.

Collier (1969), on reviewing the subject, concludes that since aspirin, given at the same dose level of sodium salicylate produces a lower blood level of unsubstituted salicylate yet still exhibits analgesic activity, it must be acting in its own right. This, however, overlooks the possibility that it may be a greater ability of aspirin to get to the site of action that makes it more effective.

Several years ago, Lester, Lolli, and Greenberg (1946) reported that the binding power of plasma protein for free salicylate was much greater than for acetylsalicylate. While they, too, felt that aspirin

was unique in action, it seems likely that the limited degree of binding exhibited by this compound would only act to enhance its efficiency.

Again condisering the results of this study, it seems probable that the observed effects on growth are due to a combination of effects rather than action at a single or very limited number of sites. This leaves us with the possibility that aspirin, by coincidence, is not only more potent than other salicylates in the management of pain, fever, and inflammation but is also more effective in accomplishing a general effect on growth. Since salicylate, at the same concentration as acetylsalicylate, has, in every instance, the same action, but to a lesser degree, it seems more likely that the effects observed are due to a greater ability of acetylsalicylate to get to the sites involved.

Sodium acetate was shown to antagonize the effect of sodium salicylate on growth and the amount of acetate released on hydrolysis of aspirin would, in all probability, have a similar effect although this would be difficult if not impossible to measure. While the mechanism of action of sodium acetate can only be hypothesized at this time, it seems likely that it may serve as an additional substrate for the citric acid cycle, partially substituting for that which would enter via glycolysis but does not, due to possible effects of salicylate on the pyruvate dehydrogenase complex. As mentioned earlier, it appears that any dehydrogenase operating with a pyridine of flavin cofactor is susceptible to inhibition by salicylate. This, of course, also means that glycolysis itself as well as the citric acid cycle can be affected.

The growth stimulatory effect of sodium acetate alone presents a different situation. Pace et al (1967) have demonstrated that, at a

concentration of 1.22 mM, sodium acetate inhibited growth of human skin cells (NCTC 2414), HeLa cells, and human lung cells in culture; however, at the same concentration, it stimulated growth in mouse fibroblasts (Strain L, NCTC 929). Since inhibition at this level was evident in all three types of human cells and stimulation occured in the mouse cells used in that study as well as those used in this present one, there is a reasonable possibility that this is a species dependant action and, for that reason, results should be assessed with caution. In respect to the concentrations used in these studies, it should be noted that sodium acetate is present in the medium NCTC 135 at a concentration of 0.37 mM; all references made to the acetate levels used here are in addition to this amount.

The effects observed could be again due to a partial substitution of the exogenous acetate for substrate made available by glycolysis; but, in this situation, instead of glycolysis being inhibited, the amount of glucose available may be the limiting factor because of high cell density. If this is the case, it might be more appropriate to consider the role of acetate as one of facilitation rather than stimulation.

In studies of this sort, care must be taken to distinguish between actual inhibition of proliferation and relative reduction in cell number due to a lethal effect the substance under investigation might have. If a lethal effect occurs, the dead cells would tend to detach from the culture vessel and, for that reason, not be counted. While the time-lapse study indicated that aspirin at a concentration of 3.3 mM was lethal to "giant" cells, it appeared that prevention of mitosis was the major effect on cells of normal morphology.

Closely related to growth is glucose utilization and lactic acid production. Fulton et al (1956) using cultures of HeLa cells and Munyon and Merchant (1959), using L Strain, have demonstrated that, under normal conditions, there is a high rate of glucose utilization and lactate production at the beginning of the logarithmic growth phase and that this falls off, at first rapidly and then more slowly as the phase progresses. This trend is reversed as the decline phase is reached. Barron (1970) has shown that this sequence is also followed by Low Line cells. A similar pattern was produced by cells exposed to aspirin at concentrations of 1.67 mM and 2.78 mM; however, the amounts of glucose utilized and lactate produced were considerably higher than could be accounted for by the growth and density effect. The current study indicated that this may also be said of sodium salicylate although the effect is less pronounced.

While the relative degree of inhibition of the various dehydrogenases involved is not known, it seems probable that an effect of the pyruvate dehydrogenase complex and the dehydrogenases of the citric acid cycle would cause a shift toward the less efficient glycolytic pathway. In addition, the uncoupling effect on oxidative phosphorylation would reduce the effectiveness of even the limited number of hydrogens made available by the cycle, thus further increasing the demand for glucose or other suitable substrates.

As was true in the growth studies, sodium acetate antagonizes the effects of sodium salicylate on glucose utilization and lactate production and, here again, the antagonism may be due to the ability of acetate to bypass the pyruvate dehydrogenase complex on entry into the citric acid cycle. If this is true, the effect of salicylate on

this complex would be of considerably greater significance than its effect on other dehydrogenases involved.

At a concentration of 2.78 mM (at day 3), exposure to aspirin resulted in the highest ratio of lactate produced to glucose utilized. High ratios were also found in cells exposed to salicylate and the combination of salicylate and acetate. Acetate alone produced a ratio that was very close to or lower than that of the controls. While this data indicates that there is, indeed, a shift toward the glycolytic pathway in the case of aspirin and sodium salicylate, as suggested above, it also implies that acetate has the opposite effect.

In all instances, the ratio of lactate to glucose was higher than could be accounted for by glucose metabolism only. While the use of other substrates is obvious, little can be said concerning them and their degree of involvement in this situation.

Another related complication is the fact that lactate can be utilized by cells in culture in some situations. Graff et al (1964), using Strain L (mouse fibroblasts) and Ehrlich ascites tumor cells, have shown that low levels of glucose, 5 mg.% or less, lactate was consumed rather than produced. Munyon and Merchant (1959) using L Strain had previously demonstrated that lactate may be utilized during the second part of the logarithmic growth phase. While the adequate use of controls allows meaningful assessment of the results in a relative sense, when one considers the total amounts of lactate and glucose involved, the possibility of lactate utilization cannot be overlooked.

The role of fat metabolism and its relationship to glucose utilization and lactate production was also studied. While it was felt

that valuable information might be obtained, the results indicate that fat deposition was variable within control as well as experimental cultures. This variability appeared to be at least partially related to immediate cell density. While it looked as though the compounds under investigation exerted some effect, segregating this from that which was due to other factors, was not possible.

The sum of the information gained during these investigations indicates that salicylate and acetate, the hydrolysis products of aspirin, did not act together to produce an effect similar in magnitude to that of aspirin on growth, glucose utilization, and lactic acid production. Salicylate's effect was less pronounced than aspirin's and the presence of acetate reduced this still further.

It was shown that aspirin and sodium salicylate tend to cause a shift toward a more glycolytic metabolism. In the case of salicylate, the presence of acetate tends to reverse this. Since glucose utilization does not appear to be inhibited to any great extent and acetate is capable of bypassing pyruvate dehydrogenase, it seems probable that inhibition of this enzyme complex is of major significance.

Several studies, including this one, have demonstrated that salicylate has the same effect as aspirin on various phenomena, but the magnitude has almost always proved to be less. It appears that aspirin may be unique in its ability to get to the site of action, but it is not necessarily unique in any other respect. The acetate released on hydrolysis would, if anything tend to reduce it's effectiveness.

SUMMARY

SUMMARY

1. A comparison was made between the effects of aspirin, sodium salicylate, a combination of sodium salicylate and sodium acetate, and sodium acetate alone, on Low Line cells *in vitro*. Growth, a glucose utilizaiton, lactic acid production and degree of fat deposition in the cells were studied. In addition, time-lapse studies of the effects of aspirin at high concentration were made.

2. The compounds under investigation produced little or no effect on growth at a concentration of 0.55 mM. While some effect could be seen at 1.67 mM, a clear-cut trend emerged at 2.78 mM. At that concentration, aspirin was most inhibitory, followed by sodium salicylate and then the combination of salicylate and acetate. Sodium acetate stimulated growth.

3. Time-lapse studies of cells exposed to 3.3 mM aspirin indicated that this concentration was lethal to "giant" cells within a short time; however, prevention of mitosis appeared to be the major effect on cells of normal morphology.

4. As was true in the growth studies, a distinct trend in glucose utilization and lactate production was demonstrated at a concentration of 2.78 mM. Exposure to aspirin resulted in the highest rates. The rates were also high when the cells were exposed to sodium salicylate, a combination of salicylate and acetate, and sodium acetate. Ratios of lactate produced to glucose utilized

followed the same pattern. Exposure to sodium acetate resulted in a ratio that was similar or moderately lower than that of the control culture.

5. The degree of fat deposition was ascertained after exposure to the various compounds, at a concentration of 2.78 mM, for a period of three days. Variability of fat content that appeared to be related to other factors (such as immediate cell density) made it difficult to interpret these results at this time.

6. It was proposed that the antagonistic action of acetate on salicylate may be due to its ability to bypass the pyruvate dehydrogenase complex on entrance into the citric acid cycle. This suggests that the effect of salicylate on this complex is of particular significance.

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