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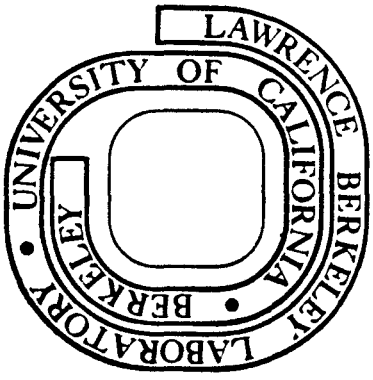

DISCLAIMER

THE CONSEQUENCES OF BISULFITE EXPOSURE IN PRIMARY CULTURES OF CHICK EMBRYO FIBROBLAST IN CULTURE.

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ABSTRACT

Bisulfite (HSO_3^-) was administered to chick embryo fibroblasts in culture. Incorporation of $HSO_3^-/SO_3^=$ was rapid and dependent on the bisulfite concentration. Hexose uptake, glucose catabolism, and membrane permeability were not affected at the concentrations examined. Nevertheless, cell growth and the rate of DNA synthesis decreased after 18 hours of exposure to $HSO_3^-/SO_3^=$ levels as low as 0.1 mM. These results indicate that the primary site of action of bisulfite is in the pathway of DNA synthesis.

INTRODUCTION

Sulfur dioxide (SO_2) is a major air pollutant and its hydrated, ionized forms, sulfite and bisulfite ions, are common food additives. Sulfur dioxide is rapidly absorbed by the blood and distributed throughout the body (YOKOYAMA et al., 1971; GUNNESON and BENTON, 1971; KOSMIDER et al., 1975). Most of the studies conducted in animals have detected no toxic effects from exposure to atmospheric levels of SO_2 (LEWIS et al., 1973; ALARIE et al., 1972). It has been argued that this may be because slowly developing, subtle effects have been overlooked, as only a limited selection of an animal's complex biological processes could be monitered (PETERING and SHIH, 1975).

Some difficulties inherent in whole animal studies which hinder the observation of effects of SO_2 on the living organism can be overcome through the use of cells in tissue culture. Although cells in culture have been available for years, they have not been put to much use with regards to the effects of sulfur dioxide on animal cells. There are only two studies of which we are aware: THOMPSON and PACE (1962) studied the gross toxic effect of high concentrations of SO_2 and its hydrated forms on established cell lines. NULSEN et al. (1974) found decreased cell viability and increased RNA and protein synthesis in murine embryonic fibroblasts and peritoneal macrophages exposed to 20 and 40 ppm SO_2 for 3 min.

Many factors can affect the actual amount of SO_2 that cells in culture are exposed to when SO_2 gas is passed over them. The interaction of SO_2 gas with cells in an aqueous environment is equivalent to the reactions of the hydrated forms of SO_2, sulfite and bisulfite (PETERING and SHIH, 1975). The amount of hydrated SO_2 in culture medium depends on the concentration of SO_2, duration of exposure, surface area of the cells, and the volume and composition of the medium.

With the exception of cells along the respiratory tract, most cells in the body are not exposed to SO_2 gas. An investigation of the effects of the hydrated forms of SO_2 on cells in culture more closely parallels the conditions of SO_2 exposure to whole animals and at the same time allows for more precise measurements at a cellular level of dose levels and metabolic responses.

We report on such an investigation into the effects of a wide range of $HSO_3^-/SO_3^=$ concentrations on the metabolism of normal chick embryo fibroblasts in culture. The intracellular pool of sulfur derived from ^{35}S-labelled $HSO_3^-/SO_3^=$ in the medium is fully labelled within 120 min. The size of this pool is directly proportional to the concentration of $HSO_3^-/SO_3^=$ in the surrounding medium. Exposure to low level $HSO_3^-/SO_3^=$ does not affect intermediary glucose metabolism, hexose uptake, or membrane permeability. However, cell growth and DNA synthesis are decreased as $HSO_3^-/SO_3^=$ concentration is increased.

Materials and Methods

[U-14C]glucose (specific activity 0.306 Ci/mmole) was obtained from Amersham. [3H]2-deoxyglucose (2DG), [3H]thymidine, [3H]mannitol, and $Na_2{}^{35}SO_3$ (specific activity 10 Ci/mmole, 20.8 Ci/mmole, 2.71 Ci/mmole, 0.214 Ci/mmole respectively) were obtained from New England Nuclear. Sodium bisulfite was obtained from Mallinckrodt.

Primary cultures were prepared from 10 day old chick embryos, as previously described (REIN and RUBIN, 1968; BISSELL et al., 1973). Embryos were decapitated, minced and washed with Tris-saline buffer, and digested with 0.25% trypsin. After 15 minutes the enzymatic action of trypsin was stopped when the suspended cells were poured into a solution of 2/3 cold medium 199 (Grand Island Biological Company) and 1/3 calf serum. The cells were centrifuged and resuspended in medium 199 containing 2% tryptose phosphate broth, 1% calf serum, and 1% heat inactivated chicken serum. Primary cultures were plated at a cell density of 8×10^6 cells per 100 mm culture dish (Falcon) in 12 1/2 mls of medium. After incubation for 4 days (at 38-39°C, 5% CO_2 (in air) atmosphere, pH of medium 7.3-7.4) the cells were trypsinized and secondary cultures were prepared. Cells were seeded at 5×10^5 cells per 35 mm culture dish in 2.5 ml of

medium 199 containing 2% tryptose phosphate broth, 2% calf serum, and 1% heat inactivated chicken serum. In experiments lasting longer than 73 hours after the secondary seeding, the medium was replaced after 48 hours.

Aqueous bisulfite concentrations were determined by the method of SEARINGELLI et al. (1967). Samples of serial bisulfite concentrations were prepared using double distilled water, medium 199, and medium 199 + 3% serum. To 1 ml of sample, 1 ml of 0.6% sulfamic acid was added After 10 min 2 ml 0.2% formaldehyde, 5 ml pararosaniline reagent B and 16 ml double distilled water was added. The absorbance at 575 nm was observed after 30 min.

Serial aqueous stock solutions of sodium bisulfite were prepared fresh for each experiment and added to the media (10 ul/m.). For overnight exposure the bisulfite was added from the stock solution 18 hours before the experiment.

Uptake experiments were performed 48 hours after the secondary cultures were seeded. Measurements of [3H]mannitol and [3H]2DG were performed as described previously (DOLBERG et al., 1975). The measurements of [3H]thymidine and $Na_2{}^{35}SO_3$ uptake also were as above except that in addition trichloroacetic acid soluble pools were measured.

Radioactivity of an aliquot of each cell sample was measured in a Packard Tri-Carb scintillation counter, model 3375, after addition of 15 ml Aquasol 2 (New England Nuclear). Protein content of an aliquot of each cell sample was measured by the method of LOWERY et al. (1951) using the Technicon Autoanalyzer II.

For [U-14C] glucose and $Na_2{}^{35}SO_3$ kinetic experiments, 1 ml of medium 199 per plate was added 1 hour prior to addition of the radioactive medium. 30' or 60' after the addition of the radioactivity, the medium was removed. The cells were washed 3 times with cold Hanks buffer containing unlabeled glucose and/or sodium bisulfite and killed with 80% methanol. The third wash contained 1/3 the usual salt concentration which reduced the salt interference with chromatographic analysis. The intermediary labeled metabolites were analyzed by autoradiography and two dimensional paper chromatography as described by BISSELL et al. (1973) and BASSHAM et al. (1974).

Cell number was determined using a Coulter Electronic cell counter.

Results

Serum proteins have been shown to bind $HSO_3^-/SO_3^=$ (GUNNESON and BENTON, 1971). It was therefore important to measure the availablility of free $HSO_3^-/SO_3^=$ under culture conditions. Table I indicates that the availability of free bisulfite ion is not decreased in medium 199 with 3% serum.

TABLE I

UNBOUND $HSO_3^-/SO_3^=$ IN DIFFERENT MEDIA

$[HSO_3^-/SO_3^=]$ (μM)	$ABSORBANCE_{575}$		
	water	medium 199	medium 199 + 3% serum
0	.03	.055	.10
50	.16	.11	.20
100	.23	.17	.22
200	.52	.34	.43
500	.53	.56	.69

Experiments were performed as described in Methods.

Exposure of secondary chick embryo cells in culture to different concentrations of $H^{35}SO_3^-/^{35}SO_3^=$ in medium 199 for different time periods indicated that uptake of $HSO_3^-/SO_3^=$ was rapid and concentration dependent. The intracellular pools of ^{35}S were directly proportional to $HSO_3^-/SO_3^=$ levels in the surrounding medium (Fig. 1). The TCA-soluble pools were saturated after 120'. Analysis of the cellular products, after exposure to $H^{35}SO_3^-/^{35}SO_3^=$, by 2-dimensional paper chromatography and autoradiography indicated that the bisulfite was not metabolized further during the course of the labeled experiments (60').

Overnight exposure of secondary cultures of chick embryo fibroblasts to 10 mM $HSO_3^-/SO_3^=$ caused morphological alterations, and increasing concentrations of $HSO_3^-/SO_3^=$ decreased cell viability. At concentrations less that 100 μM the cells seemed to recover one day after addition of $HSO_3^-/SO_3^=$ (Fig. 2).

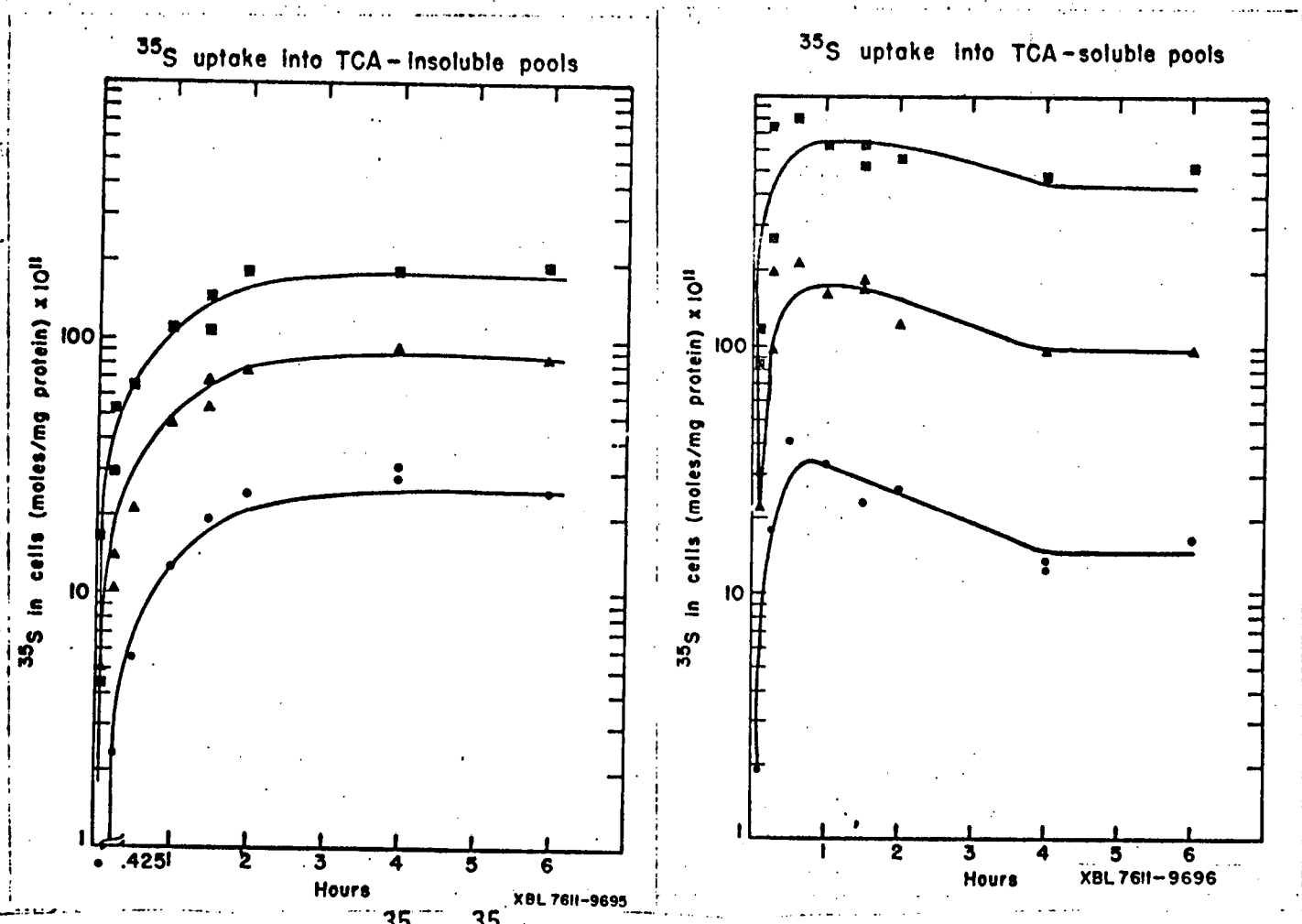

Figure 1. Uptake of $H^{35}SO_3^-/^{35}SO_3^=$ into chick embryo fibroblasts. (a) TCA-insoluble pools (b) TCA-soluble pools. Concentration of $HSO_3^-/SO_3^=$ in medium 199: (■) 500 μM, (▲) 100 μM, (●) 20 μM. Average of duplicate samples.

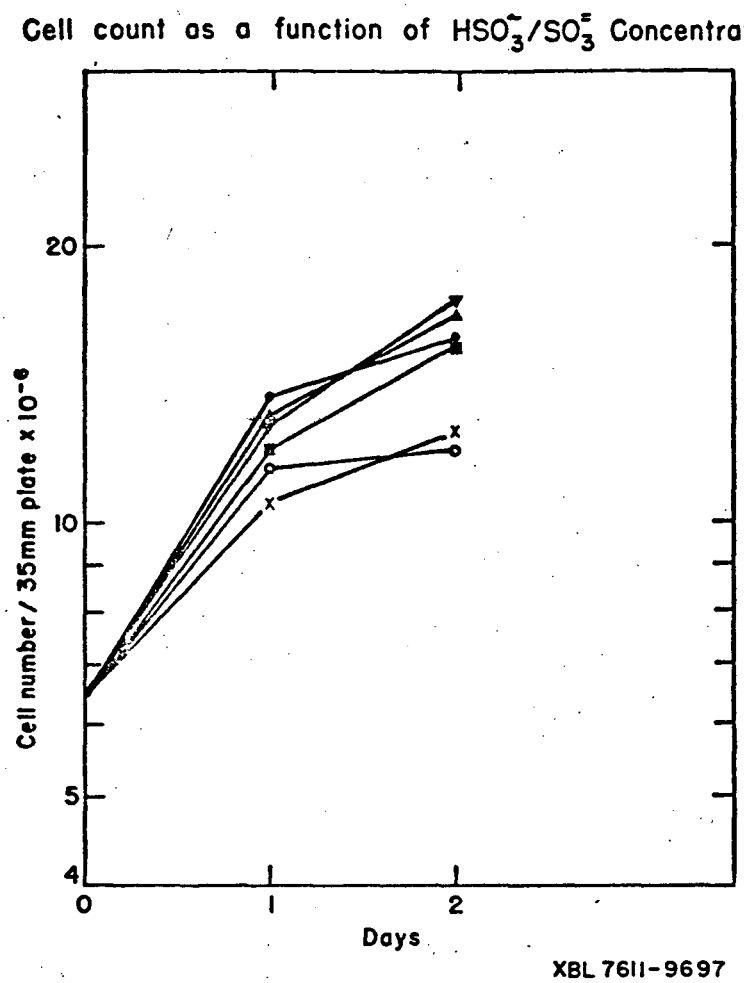

Figure 2. Effect of $HSO_3^-/SO_3^=$ on cell viability. Concentration of $HSO_3^-/SO_3^=$ in medium: (●) control, (▲) 10 μM, (■) 50 μM, (▼) 100 μM, (○) 500 μM, (x) 1000 μM. Average of duplicate samples of two experiments.

To determine the effect of $HSO_3^-/SO_3^=$ on the rate of DNA synthesis, the rate of[3H]thymidine incorporation into the TCA-insoluble pool was measured (BISSELL et al., 1972). We found that the rate of DNA synthesis was decreased as $HSO_3^-/SO_3^=$ concentration was increased (Table II).

TABLE II

INCORPORATION OF 3H-THYMIDINE INTO CHICK EMBRYO FIBROBLASTS EXPOSED TO $HSO_3^-/SO_3^=$ OVERNIGHT

Overnight $HSO_3^-/SO_3^=$ Exposure (μM)	Incorporation of 3H-thymidine	% reduction in incorporation
0	38800	-
50	38800	0
100	33400	14
500	24100	38
1000	18600	52

Experiments were performed as described in Methods.

After 18 hour exposure to $HSO_3^-/SO_3^=$, the lowest toxic concentration was 100 μM. The TCA-soluble pools were the same for treated and untreated cells indicating that the decreased uptake was a result of a lowered rate of synthesis and not a result of lowered [3H]thymidine pools in the cell.

The effect of $HSO_3^-/SO_3^=$ on membrane permeability was measured by the rate of [3H]2DG uptake and [3H]mannitol diffusion into the cell (DOLBERG et al., 1975). Mannitol is taken up by non-carrier mediated diffusion and 2DG is transported by carrier mediated diffusion in these cells (WEBER, 1973). The rate of uptake of these sugars was unaffected after overnight exposure to $HSO_3^-/SO_3^=$, up to the highest concentration examined (1 mM).

Analysis of the levels of various glycolytic and TCA cycle metabolites derived from [U-14C]glucose using two-dimensional paper chromatography and autoradiography (BISSELL et al.,1973, and BASSHAM et al., 1974) showed no differences between exposed cells and controls. The metabolites separated on paper included intermediates of the glycolytic pathway (such as hexose mono phosphates, fructose di-phosphate, 3-phosphoglyceric acid, and lactic acids), intermediates of the oxidative pentose shunt, inter-

mediates of glycogen synthesis (uridine-di-phospho glucose), tricarboxylic acid intermediates (citrate and malate), and amino acids derived from them (glutamate and aspartate) and energy metabolites. [U-14C] metabolite pool levels were the same after 30' and 60' administration of [U-14C] glucose following 1 hour and 18 hour exposure to 100 μM $HSO_3^-/SO_3^=$.

Discussion

Cell viability studies reported here indicate that secondary chick embryo cells in culture are more sensitive to $HSO_3^-/SO_3^=$ than has been reported for established cell lines (THOMPSON and PACE, 1962). This may be a result of differences in the metabolism of established cell lines versus primary cultures. However, some of the differences may be due to the availability of free $HSO_3^-/SO_3^=$ to the cells under the experimental conditions used.

The levels of $HSO_3^-/SO_3^=$ at which toxic effects were observed in our experiments were comparable to blood levels found in rabbits following 62 hour exposure to 23-24 ppm SO_2 gas (GUNNESON and BENTON 1971), although in the blood almost all of the $HSO_3^-/SO_3^=$ is bound to serum proteins.

The lack of effect of $HSO_3^-/SO_3^=$ on membrane permeability, hexose uptake, and glucose catabolism indicate that the primary effects of $HSO_3^-/SO_3^=$ must involve other cellular functions. The observed decrease in DNA synthesis and cell viability following exposure to $HSO_3^-/SO_3^=$ indicates that the primary site of action interferes with the pathway of DNA synthesis.

Our findings are consistent with *in vitro* studies which indicated alteration of nucleic acids following $HSO_3^-/SO_3^=$ exposure. Bisulfite has been shown to catalyze the conversion of cytosine derivatives to uracil compounds *in vitro* (HAYATSU et al. 1970). Cytidine residues in E. coli formylmethionine-tRNA were converted to uridine residues by bisulfite (GODDARD et al. 1972). The concentrations of bisulfite in these experiments, however, were much higher than in our study. *In vitro* studies at lower concentrations of bisulfite (10^{-3}M) brought about cleavage of phosphodiester bonds in DNA (HAYATSU and MILLER 1972). Future investigations will help determine if the mechanisms by which bisulfite exerts its action on chick cells in culture are similar to those deduced from *in vitro* studies.

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