[CANCER RESEARCH 43, 1251–1255, March 1983]
0008-5472/83/0043-0000$02.00

Influence of Asbestos on the Uptake of Benzo(*a*)pyrene and DNA Alkylation in Hamster Tracheal Epithelial Cells[1]

Alan Eastman,[2] Brooke T. Mossman, and Edward Bresnick

Departments of Biochemistry [A. E., E. B.] and Pathology [B. T. M.], and The Vermont Regional Cancer Center, The University of Vermont College of Medicine, Burlington, Vermont 05405

ABSTRACT

The objective of these experiments was to understand the mechanism of cocarcinogenicity of asbestos and polycyclic aromatic hydrocarbons. Benzo(*a*)pyrene [B(a)P] was coated onto crocidolite or chrysotile asbestos fibers, resuspended in serum-free medium, and added to cultures of hamster tracheal epithelial cells. The fibers markedly enhanced cell uptake of B(a)P. Although considerable metabolism occurred, approximately 40% of the applied B(a)P was retained by the cells after 8-hr incubation as opposed to 5% after incubation with B(a)P in the absence of asbestos. The hydrocarbon-containing medium was replaced by fresh medium. Four days later, approximately 3% of the B(a)P that had been applied when adsorbed to asbestos was still persistent in cells as compared to 0.5% in cells treated with B(a)P alone. DNA from hamster tracheal epithelial cells was purified, and the amount of B(a)P alkylation was assessed. At 8 hr, the extent of alkylation after treatment of the cells with either B(a)P or B(a)P:asbestos was similar. However, the retained unmetabolized B(a)P was subsequently metabolized and contributed to further alkylation so that the B(a)P-asbestos treated cells demonstrated considerably higher levels of alkylation throughout the 4-day posttreatment period. None of these effects was observed if asbestos was added 1 hr before the addition of B(a)P. The enhanced uptake of B(a)P and subsequent additional alkylation of DNA might represent a mechanism of asbestos-induced cocarcinogenesis.

INTRODUCTION

The term "asbestos" represents a group of silicate minerals that have extensive commercial use. Exposure to asbestos in certain occupations is recognized as a major health hazard. Both fibrotic lung disease and an increased frequency of mesothelioma, an otherwise rare cancer, occur in asbestos workers (15). Perhaps of greater significance with respect to absolute numbers of afflicted people is an increased risk of bronchogenic carcinoma; its frequency among asbestos workers who smoke is 80 to 92 times that of the general nonsmoking population (14, 16). This synergistic effect has been attributed to cocarcinogenic interactions between asbestos and the carcinogenic principals of tobacco smoke. Experimental studies also support this concept. For example, intratracheal instillation of asbestos into rodents produces bronchogenic neoplasms only when PAH[3] are adsorbed to or mixed with the fibers (8, 17). Although hydrocarbons alone are carcinogenic, more tumors are produced when the chemicals are administered on a variety of fibers and particles.

Particulates in the atmosphere serve as condensation nuclei for environmental PAH (4) and can prevent photooxidative degradation of the chemicals (3). We hypothesized that, under these circumstances, increased uptake and retention of PAH by cells of the respiratory tract might occur. To test this hypothesis, we used a cloned hamster tracheal epithelial cell line (HTE-B) well characterized with respect to B(a)P metabolism, DNA alkylation, and repair (1, 2). We now report on the modulation of the interaction of B(a)P with HTE-B cells when B(a)P is coated on 2 types of asbestos, crocidolite and chrysotile.

MATERIALS AND METHODS

Cell Cultures. HTE-B cells were maintained as described previously (11). Confluent monolayers in 150-sq cm flasks (approximately 2×10^7 cells) were incubated with 10^{-7} M [^{3}H]B(a)P (40 Ci/mmol; Amersham Corporation, Arlington Heights, Ill.) for 1 to 8 hr in MEM (Grand Island Biological Company, Grand Island, N. Y.) without serum. During the posttreatment period, cells were maintained in MEM with 2% fetal calf serum. Little cell detachment was observed during this period. Cells were harvested by scraping with a rubber policeman, DNA was purified by hydroxyapatite, and its specific radioactivity was determined. Any continuing DNA replication was corrected by the prior addition of [^{14}C]thymidine (2). The conversion of B(a)P to water-soluble metabolites in medium was assessed also (2), and the residual B(a)P in the cells was calculated from an aliquot of the sonicated cell preparation prior to hydroxyapatite chromatography.

Crocidolite and chrysotile asbestos (both International Union Against Cancer reference samples) were suspended in benzene, and an amount of [^{3}H]B(a)P was added. For coating the fibers, a 25% (crocidolite) or 50% (chrysotile) excess of [^{3}H]B(a)P was used. This permitted correction for the loss of B(a)P during transfer to medium and thereby ensured that the final concentration of B(a)P in medium was approximately 10^{-7} M for both experiments, with and without asbestos. The benzene was evaporated under a stream of nitrogen, and the B(a)P-coated fibers were resuspended in serum-free medium. Aliquots were centrifuged to assess the proportion of B(a)P-coated on the fibers. It was routinely found that >90% of the hydrocarbon pelleted with the asbestos. The final concentrations of crocidolite and chrysotile in medium were 10 and 3 μg/ml, respectively, (2 and 0.6 μg/sq cm Petri dish, respectively). These doses cause no inhibition of normal cell growth (7). In several experiments, asbestos was added to cultures and incubated for 1 hr, and the medium was then replaced with MEM containing [^{3}H]B(a)P. The majority of asbestos remained attached to the cell monolayer during this medium change.

Autoradiography. At 1 hr after addition of [^{3}H]B(a)P alone or coated on asbestos, medium was removed from cultures, and monolayers were rinsed twice with fresh medium before fixation in 2.5% glutaraldehyde. After 18 hr, the cells were postfixed in 10% osmium tetroxide, dehydrated, and embedded *in situ* in Epon (11). Sections (≃1 μm thick)

[1] Supported by National Cancer Institute Grants CA 23514 and CA 30670, and Grant N01CP 33360 from the National Institute for Occupational Safety and Health.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; HTE-B, hamster trachea epithelial cells, clone B; B(a)P, benzo(*a*)pyrene; MEM, minimal essential medium.

Received July 30, 1982; accepted December 8, 1982.

were prepared with an ultramicrotome and mounted on glass slides for autoradiography as described previously (9).

RESULTS

Coating of Asbestos Fibers with [^{3}H]B(*a*)P. After evaporation of the organic solvent and resuspension of the fibers in medium, autoradiographs demonstrated even coating of the fibers by [^{3}H]B(a)P, (Fig. 1*a*). In contrast, when [^{3}H]B(a)P-coated fibers were resuspended in MEM containing 2% fetal calf serum, most of the [^{3}H]B(a)P rapidly dissociated into the medium (Fig. 1*b*). Uncoated fibers are poorly discerned in this autoradiograph. After centrifugation in serum-free MEM, 90% of the [^{3}H]B(a)P was associated with the fibers, whereas only 10 to 20% remained on asbestos in the presence of serum. Similar results were obtained with both crocidolite and chrysotile. The dissolution of B(a)P from the fibers by serum necessitated the use of serum-free medium in our experiments.

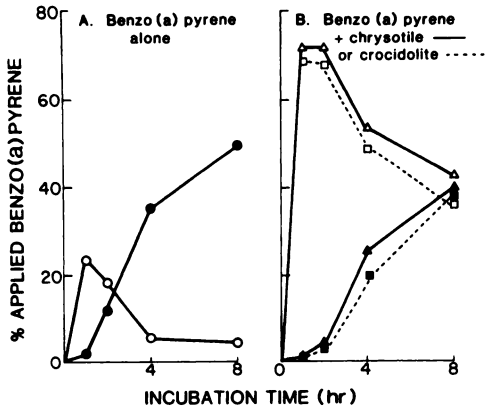

Chart 1. Uptake and metabolism of [^{3}H]B(a)P in HTE-B cells upon incubation with B(a)P alone (○, ●) or B(a)P-coated crocidolite (□, ■) or chrysotile (△, ▲). Radioactivity associated with the cell pellet (○, □, △) and recovered as aqueous methanol-soluble metabolites (●, ■, ▲) were assessed. Values represent means of up to 5 separate determinations.

Uptake of [^{3}H]B(*a*)P by Cells. After HTE-B cells were incubated with [^{3}H]B(a)P-coated asbestos fibers, radioactivity was almost exclusively associated with cells and no longer associated with asbestos fibers (Fig. 2). The uptake process was quantitated by analyzing the [^{3}H]B(a)P associated with the cell pellet after harvesting. In the absence of asbestos fibers, a maximum of approximately 20% of the [^{3}H]B(a)P was taken up by the cells at 1 hr, while only about 5% was retained in the cells at 8 hr (Chart 1*A*). In comparison, >70% uptake of the B(a)P from coated crocidolite and chrysotile occurred within the first hr of incubation, and about 40% of the total applied PAH was retained in the cell at 8 hr (Chart 1*B*). This enhanced uptake was attributed to deposition of the PAH-coated asbestos fibers on the cell monolayer and rapid release of B(a)P to the cells.

Metabolism of [^{3}H]B(a)P. Throughout the 8-hr uptake period, aliquots of the medium were analyzed for water-soluble metabolites of B(a)P. These include conjugates such as glucuronides but not unconjugated metabolites such as B(a)P

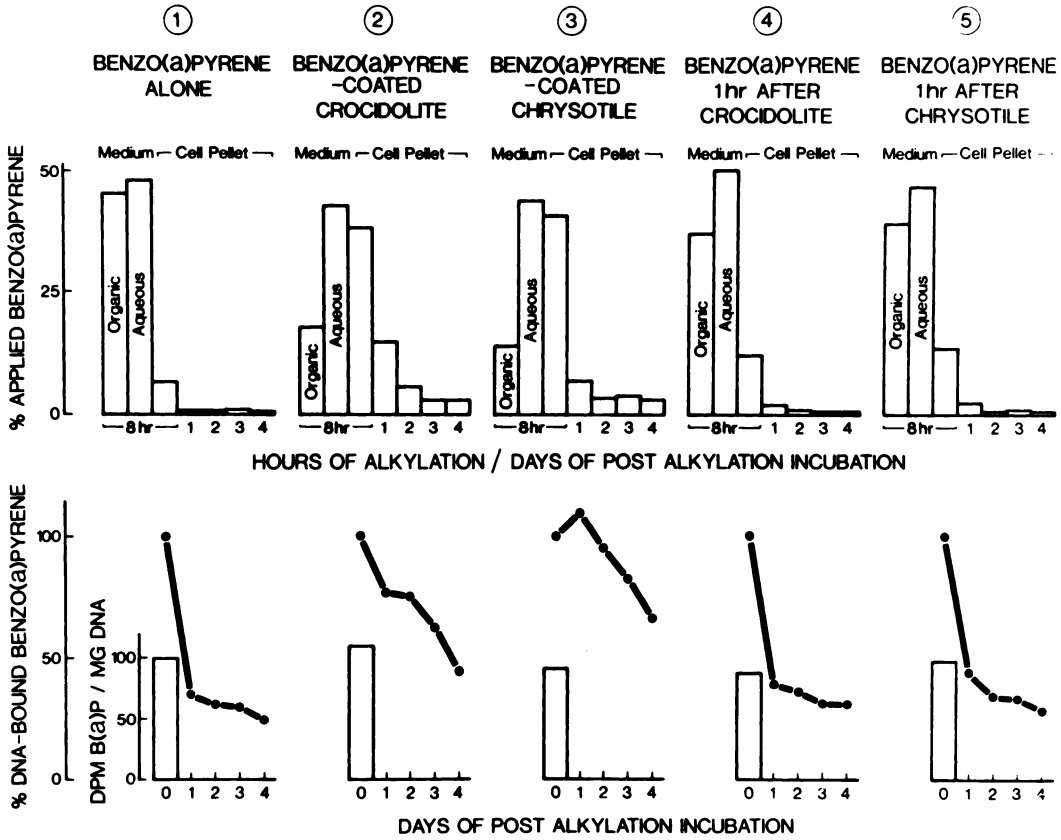

Chart 2. Fate of [^{3}H]B(a)P incubated with HTE-B cells in the presence or absence of asbestos fibers. *Top*: *first 3 boxes*, percentage of applied B(a)P in the organic or aqueous extract of medium or in the cell pellet at 8 hr. *Subsequent boxes*, remaining B(a)P in the cell pellet up to 4 days. *Bottom*: *boxes*, specific activity of purified DNA after 8-hr incubation with [^{3}H]B(a)P. Assuming this value to be 100%, *lines*, subsequent removal of DNA-bound adducts during the 4 days of posttreatment incubation.

phenols and quinones, the latter of which represented <5% of the recovered metabolites (2). The extent of metabolism was approximately equal for all treatments with about 40 to 50% of the applied [^{3}H]B(a)P recovered in the medium as aqueous metabolites. In cells exposed to B(a)P-coated asbestos, a small but consistent reduction in the level of metabolism was observed (Chart 1). The spectrum of metabolites from cells exposed to B(a)P alone was as reported previously (2) and was not altered after exposure of cells to B(a)P adsorbed to asbestos.

It was of interest to determine the ultimate fate of the residual B(*a*)P in the cells. After 8-hr incubation with B(a)P, the culture medium was replaced, and cells were maintained for up to 4 days. In the absence of asbestos, the residual cellular B(a)P declined to about 0.5% of that applied and persisted at that level throughout the 4-day posttreatment incubation (Chart 2, *Column 1*). In the presence of either chrysotile or crocidolite, some [^{3}H]B(a)P was excreted over 2 days, but about 3% of the applied radioactivity was still retained at Day 4 (Chart 2, *Columns 2* and *3*). The cells, therefore, not only took up more [^{3}H]B(a)P in the presence of asbestos but were also exposed to a significantly higher level for a considerable length of time. When B(a)P was added to the cells 1 hr after asbestos, the resultant uptake, metabolism, and persistence of B(a)P were the same as if the asbestos were not present (Chart 2, *Columns 4* and *5*).

Alkylation of DNA. DNA was purified from the HTE-B cells used in the above experiments. After an 8-hr incubation with [^{3}H]B(a)P, the specific radioactivities were similar in all treatment groups (Chart 2). Analysis of deoxyribonucleoside-bound adducts by high-pressure liquid chromatography showed similar spectra of adducts compared to those described previously (2) and did not change with asbestos treatment. A marked difference, however, was seen in the rates of DNA repair during posttreatment incubation. In the absence of asbestos, about 60% of the adducted B(a)P was removed in 24 hr followed by a slow second stage of repair (Chart 2, *Column 1*). When B(a)P was proffered to the cells on fibers, the subsequent repair was much slower. In the case of chrysotile, alkylation levels even increased after the medium change (Chart 2, *Column 3*). This is indicative of retained B(a)P metabolizing and contributing to continued alkylation. When asbestos and B(a)P treatments were separated by 1 hr, the rate of repair appeared to be identical to that in the absence of the fibers.

DISCUSSION

B(*a*)P is a major carcinogenic PAH in cigarette smoke and has been implicated in the enhanced bronchogenic carcinoma observed in both asbestos workers and smokers in the general population. The experiments reported here demonstrate that asbestos mediates a significant increase in cellular uptake of B(a)P when the PAH is coated on the fibers. Our results are supported by studies of Lakowicz and Bevan (5) and Lakowicz and Hylden (6), who monitored by fluorimetry the rapid transfer of B(a)P into lipid micelles and microsomes when the hydrocarbon was adsorbed to a variety of particles and fibers. However, our studies have the distinct advantage of using an intact cell system and "target" cells of both asbestos and cigarette smoke. This model has facilitated study of the fate of B(a)P transmitted to cells. The HTE-B cells are capable of metabolizing an increased load of B(a)P but, after 8 hr, 40% of the B(a)P introduced on asbestos is still retained in the cells, as opposed to 5% in the absence of asbestos. After 4 days of post-alkylation incubation, the B(a)P:asbestos-treated cells still retain about 3% of the applied radioactivity, as opposed to <0.5% in control cells.

Although asbestos caused a 4-fold increase in uptake of B(a)P at 1 hr, *i.e.*, 80% of the applied B(a)P, all treatments resulted in about 50% conversion of B(a)P to water-soluble metabolites by 8 hr. The asbestos treatment, therefore, resulted in exposure of the cells to an additional 30% of the applied B(a)P. The 6-fold increase in persistent radioactivity after 4 days, therefore, probably represents a change in metabolism such that a greater proportion of metabolites becomes associated with macromolecules. An additional observation was an apparent inhibition of removal of adducts in experiments with B(a)P-coated fibers. Recently, we have observed the same phenomenon after incubation of HTE-B cells with higher concentrations of B(a)P ($\simeq 10^{-6}$ M); indeed, the apparent repair rate for B(a)P-coated chrysotile almost superimposes upon a curve produced at 10^{-6} M B(a)P (2). However, in both cases, this can be attributed to the metabolism of residual B(a)P retained by the cells after the 8-hr alkylation period. The overlapping kinetics of formation and removal of adducts makes it difficult to ascertain whether there is indeed an inhibited repair process. However, all experiments with B(a)P-coated asbestos demonstrate that the cells respond as if they were exposed to a considerably higher dose of B(a)P compared to incubation with B(a)P alone or incubation with asbestos before B(a)P.

Asbestos is a documented carcinogen in humans. Many particulates have also been shown to be cocarcinogenic in experimental models of respiratory carcinogenesis (10, 13). In preliminary studies, we have observed that both hematite and kaolin enhance uptake of B(a)P into HTE-B cells but not as effectively as does asbestos. This is consistent with their cocarcinogenic capability in hamster tracheal grafts (10) and their relative enhancement, compared to asbestos, of B(a)P uptake into microsomes (5, 6).

The mechanisms of asbestos-induced cocarcinogenesis appear to be complex. When administered alone to tracheal organ cultures and grafts, asbestos acts as a classical tumor promoter by induction of inflammatory, proliferative, and metaplastic alterations (reviewed in Ref. 12). The hyperplasia of basal cells, which are the presumed progenitors of bronchogenic carcinoma, after exposure to asbestos increases the probability for replication on a DNA template damaged by a chemical carcinogen such as B(a)P. It can be envisaged that exposure to asbestos would also result in an increased target size, *i.e.*, absolute quantity of DNA, and hence a greater probability for initiation by B(a)P. The work described here shows increased cell uptake and alkylation of DNA by B(a)P when the hydrocarbon is coated on asbestos. This may represent another important mechanism of cocarcinogenesis.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Marie Perron and Lucy Jean.

REFERENCES

1. Eastman, A., Mossman, B. T., and Bresnick, E. Formation and removal of benzo(*a*)pyrene adducts of DNA in hamster tracheal epithelial cells. Cancer Res., *41:* 2605–2610, 1981.
2. Eastman, A., Mossman, B. T., and Bresnick, E. Modulation of the interaction of benzo(*a*)pyrene with a hamster tracheal epithelial cell line. Carcinogenesis (Lond.), *3:* 1283–1287, 1982.
3. Korfmacher, W. A., Natusch, D. F. S., Taylor, P. D., Mamantov, G., and Wehry, E. L. Oxidative transformations of polycyclic aromatic hydrocarbons adsorbed on coal flu ash. Science (Wash. D. C.), *207:* 763–765, 1980.
4. Krstalovic, A. M., Rosie, D. M., and Brown, P. R. Distribution of some atmospheric polynuclear aromatic hydrocarbons. Am. Lab. (Fairfield, Conn.), *9:* 11–18, 1977.
5. Lakowicz, J. R., and Bevan, D. R. Effects of asbestos, iron oxide, silica and carbon black on the microsomal availability of benzo(*a*)pyrene. Biochemistry, *18:* 5170–5176, 1979.
6. Lakowicz, J. R., and Hylden, J. L. Asbestos-mediated membrane uptake of benzo(*a*)pyrene observed by fluorescence spectroscopy. Nature (Lond.), *275:* 446–448, 1978.
7. Landesman, J. M., and Mossman, B. T. Induction of ornithine decarboxylase in hamster tracheal epithelial cells exposed to asbestos and 12-*O*-tetradecanoylphorobol-13-acetate. Cancer Res., *42:* 3669–3675, 1982.
8. Miller, L., Smith, W. E., and Berliner, S. W. Tests for effect of asbestos on benzo(*a*)pyrene carcinogenesis in the respiratory tract. Ann. N. Y. Acad. Sci., *132:* 489–500, 1965.
9. Mossman, B. T. Autoradiography for determination of DNA synthesis in the hamster bladder epithelium. T. C. A. Man., *3:* 363–365, 1977.
10. Mossman, B. T., and Craighead, J. E. Comparative cocarcinogenic effects of crocidolite asbestos, hematite, kaolin and carbon in implanted tracheal organ grafts. *In:* A. Critchlow (ed.), Inhaled Particles, Vol. 5, London: Pergammon Press, pp. 572–578, 1982.
11. Mossman, B. T., Ezerman, E. B., Adler, K. B., and Craighead, J. E. Isolation and spontaneous transformation of cloned lines of hamster tracheal epithelial cells. Cancer Res., *40:* 4403–4409, 1980.
12. Mossman, B., Light, W., and Wei, E. Asbestos: mechanisms of toxicity and carcinogenicity in the respiratory tract. Ann. Rev. Pharmacol. Toxicol., *23:* in press, 1983.
13. Nettesheim, P. Respiratory carcinogenesis studies with the Syrian Golden Hamster: a review. Prog. Exp. Tumor Res., *16:* 185–200, 1972.
14. Selikoff, I. J., Hammond, E. C., and Chung, J. Asbestos exposure, smoking and neoplasia. J. A. M. A., *204:* 104–110, 1968.
15. Selikoff, I. J., Lilis, R., and Nicholson, W. J. Asbestos disease in United States shipyards. Ann. N. Y. Acad. Sci., *330:* 295–311, 1980.
16. Selikoff, I. J., Seidman, H., and Hammond, E. C. Mortality effects of cigarette smoking among amosite asbestos factory workers. J. Natl. Cancer Inst., *65:* 507–513, 1980.
17. Shabad, L. M., Pylev, L. N., Krivosheera, L. V., Kulagina, T. F., and Nemenko, B. A. Experimental studies on asbestos carcinogenicity. J. Natl. Cancer Inst., *52:* 1175–1187, 1974.

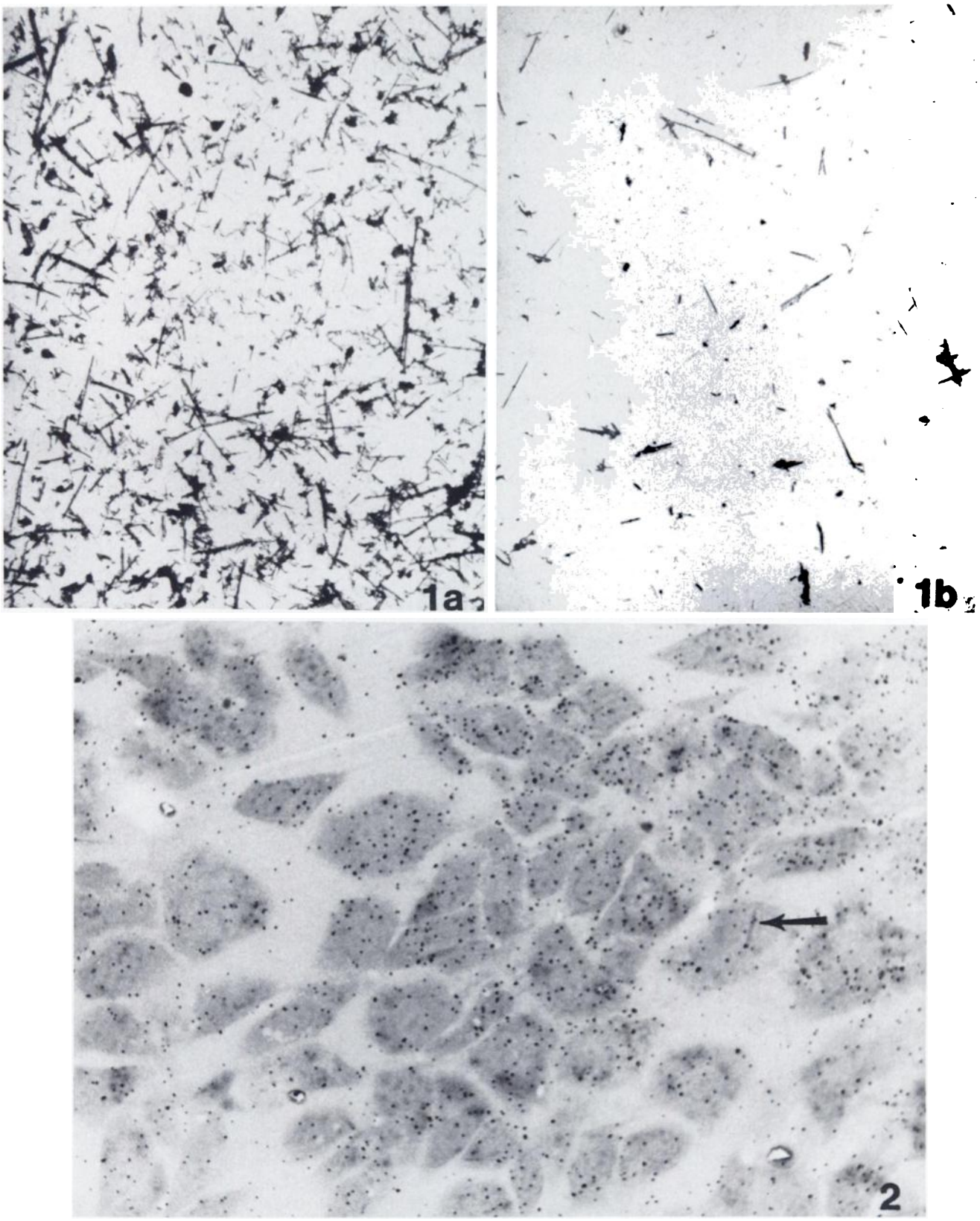

Fig. 1. Autoradiograph of [^{3}H]B(*a*)P-coated crocidolite after resuspension in either medium without serum (*A*) or medium with 2% fetal calf serum (*B*). Note dissociation of label (*background*) from fibers in *B*. × 600.

Fig. 2. Autoradiography of HTE-B cells after incubation for 1 hr with [^{3}H]B(*a*)P-coated crocidolite. B(*a*)P has dissociated from fibers (*arrow*) and is intracellular. Toluidine blue, × 2000.