TABLE I. Potentiation of Local Shwartzman Reaction by Trypsin.

| | .025 mg intraderm. | | | .012 mg intraderm. | | | .006 mg intraderm. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Positives | | | Positives | | | Positives | |
| | No. of cases | Without trypsin | With trypsin | No. of cases | Without trypsin | With trypsin | No. of cases | Without trypsin | With trypsin |
| *E. coli* endotoxin | 9 | 5 | 8 | 8 | 3 | 4 | 2 | 0 | 0 |
| *P. vulgaris* polysaccharide | 10 | 9 | 10 | 11 | 6 | 9 | 10 | 1 | 5 |
| Total | 19 | 14 | 18 | 19 | 9 | 13 | 12 | 1 | 5 |

might be differentiated and evaluated. The importance of this is exemplified in other reactions which are potentiated or activated by trypsin(10-15).

It is also worthy of note that heparin, which inhibits the Shwartzman Phenomenon(16) also inhibits action of trypsin and serum tryptase(17).

*Summary.* 1) Trypsin potentiates the effect of skin preparatory substances in elicitation of the Shwartzman Phenomenon. 2) In combination with trypsin, the skin reacts to a greater degree and with lower concentrations of the preparatory factor.

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## Tissue Culture Bioautographic System.* (25651)

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A bioautographic system under agar has been developed for paper chromatographic analysis of cytotoxic agents *in vitro*. Applications of paper chromatography and bioassay methods in fermentation beer analyses are described.

*Materials and methods.* Eagle's KB line of human epidermoid carcinoma cells was maintained and harvested as reported previously (1). Cells grown in suspension culture provide a more convenient inoculum than bottle cultures and studies of submerged growth will be reported elsewhere. Cell content of bottle cultures was determined as protein concentration(2); suspension culture populations were determined by hemacytometer count.

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Two milligrams of cell protein (or approximately 4 x $10^6$ cells) in 10 ml of Eagle's medium(3) containing 10% calf serum were added to a flat-bottomed Petri dish. Plates were then incubated at 37°C, 80-84% humidity and 8-10% $CO_2$ in air. Under these conditions, generation time was 18-24 hours. After 18 hours' incubation, supernatant medium was decanted and cell sheet was overlaid with 7 ml of Eagle's medium containing vitamins and amino acids at double strength; adjusted to pH 7 with $NaHCO_3$; 10% calf serum; 0.1% Difco Bacto-Peptone and 0.7% Bacto Noble agar (previously washed exhaustively with distilled water). KB cells under agar had a generation time of approximately 40 hours in above medium. When the agar had solidified, a section of developed papergram was laid on the surface and incubation was continued for 24 hours. When constructing bioautograms it is convenient to divide the chromatogram from origin to solvent front into five equal parts which then can easily fit across Petri dishes. After incubation agar and papergram were floated from the plates in a stream of Earle's salts (minus bicarbonate). Usually, washing the sheet is sufficient to remove cells which have been injured by cytotoxic agents from the chromatogram. When it is desirable to emphasize the zone of inhibition, addition of 10 ml of 0.0125% trypsin in saline and 2-3 minutes' incubation at room temperature is sufficient to cause complete removal of areas of drug-injured cells, since these have a diminished capacity to remain attached to glass and are, therefore, more susceptible to tryptic action than are untreated cells. Experiments with streptovitacin A(4) have shown that the agar technic described above can also be used for disc-plate assay. Aqueous solutions of agent were pipetted onto 13 mm discs which were incubated on the agar surface at 37°C overnight. Agar and discs were removed and the cell sheet was treated as described above before measuring zone size.

*Results.* Chromatograms of known antitumor agents and fermentation beers were developed in 6 solvent systems listed in Table I. Papergram patterns of actinomycin D with KB cells and *Bacillus subtilis* as test organisms are presented in Figs. 1 and 2. These data show that actinomycin bioactivity is identical when measured in 2 assay systems. Successful papergrams have also been obtained with mitomycin C, streptovitacin family, echinomycin family and fermentation liquors containing unidentified cytotoxic agents. Papergrams of some fermentation products show anti-KB activities while no antibacterial zones can be found. The contrast between "treated" and "untreated" areas must be great to allow quick and definite interpretation of KB bioautographs. This contrast was obtained by the use of a large inoculum as described. If desired, the contrast can be accentuated by fixing the cells with 10% formalin followed by Giemsa stain. Fig. 3 illustrates zones obtained with this procedure.

It should be noted that no inhibition of cells under agar results from application of filter paper *per se,* as evidenced by the confluency of cell sheet on each side of zone of in-

TABLE I. Solvent Systems Used in Tissue Culture Bioautographic System.

| System | Composition |
|---|---|
| I | n-butanol:water, 84:16 (v/v) |
| II | n-butanol:water, 84:16 plus 0.25% (w/v) *p*-toluenesulfonic acid |
| III | n-butanol:acetic acid:water, 2:1:1 (v/v/v) |
| IV | n-butanol:water, 84:16 (v/v), plus 2 ml piperidine/98 ml butanol:water |
| V | water-n-butanol 96:4 (v/v) |
| VI | *Idem* plus 0.25% *p*-toluenesulfonic acid |

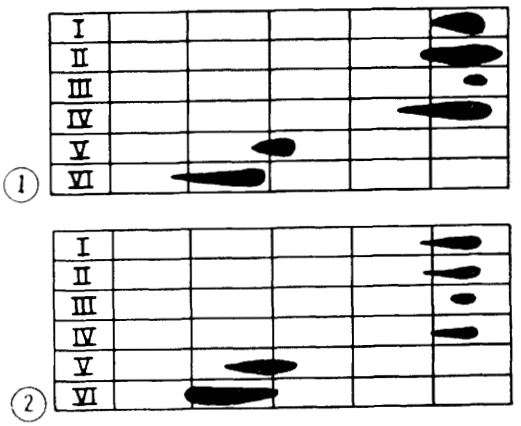


FIG. 1. Papergram pattern of actinomycin D *vs* KB cells, 1 μg spotted.

FIG. 2. Papergram pattern of actinomycin D *vs* *B. subtilis*, 0.5 μg spotted.

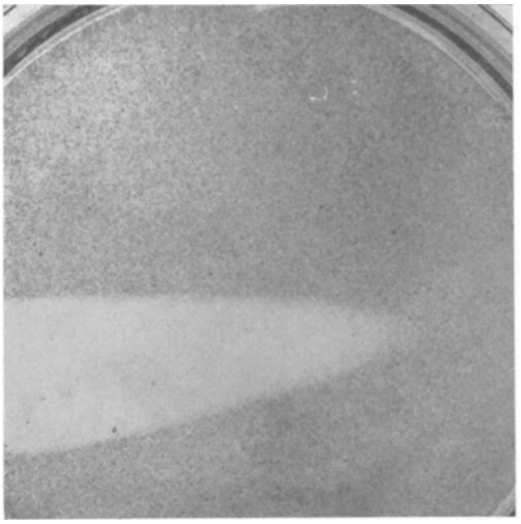

FIG. 3. Photograph of a zone of inhibition obtained by KB bioautograph procedure.

hibition. Thus, the sheet of paper placed on the agar plate shown in Fig. 3 measured approximately 1 x 3 in., whereas the zone of inhibition occurred only where a specific inhibitor was present. Each plate, therefore, serves in part as its own control. The tissue culture bioautogram system provides the only means available to identify unknown cytotoxic agents present in beers and to determine with certainty which activity of a mixture has been removed during fractionation studies.

Disc-plate assay procedure is shown in Fig. 4 with streptovitacin A. As with chromatograms, at least 2 $\mu$g of streptovitacin A per disc resulted in complete removal of cells from the surface of the Petri dish giving a zone of at least 18 mm (13 mm disc). Paper discs without agent (controls) did not injure KB cells under agar. If disc-plate assay method can be made as reproducible as tube-assay method(1), it will certainly replace the more laborious procedure (as has occurred with antibacterial and antifungal assays). The technic is most promising and is applicable to determination of chemicals and antibiotics which diffuse in agar. The method is less sensitive than the tube assay however [2 $\mu$g per disc can be detected under agar whereas 0.01 $\mu$g per ml can be detected in the tube assay(1)].

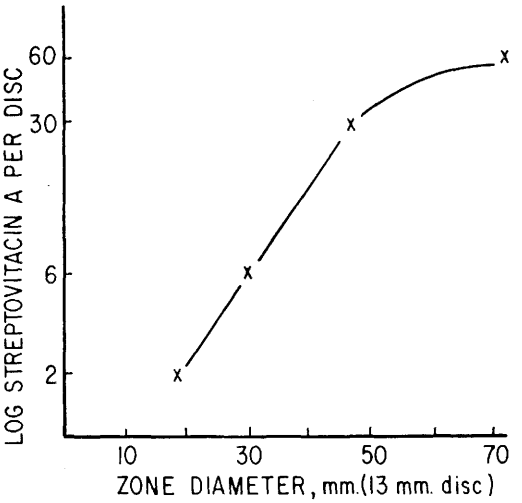


FIG. 4. Effect of streptovitacin A on KB cells in disc-plate assay procedure.

*Discussion.* The tissue culture bioautograph system has been used in these laboratories to identify cytotoxic activities in fermentation liquors. The technic allows detection of multicomponent cytotoxic activities, identification of components during purification procedures and recognition of identical activities in different beers. An experiment can be completed in 48 hours when the procedure described here is used. If the inoculum is doubled and agar and papergram are added 3-4 hours after inoculation an experiment can be completed in 24 hours. Disc-plate assays and agar papergram systems place screening for potential antitumor agents in the same category as searching for antibacterial or antifungal agents.

Agar-cell systems for assay and chromatography have been described with Ehrlich ascites carcinoma and HeLa cells(5-9) in which dye reduction was used to determine the cytotoxic response. The procedures described here should give a more distinct endpoint determination since dead and injured cells are removed while uninhibited cells remain as a confluent sheet. Cells are growing throughout the period of contact with cytotoxic agents in the KB method, whereas the dye reduction system consists primarily of resting cells. The KB system should be more sensitive with metabolic inhibitors which affect vital processes necessary for growth, division and attachment to glass, since it is entirely feasible that cells in which protein, nucleic acid or energy

metabolism is interrupted may be able to reduce a dye but unable to grow. Thus, the KB system is different from those described previously.

*Summary.* A tissue culture bioautograph system has been described which allows identification of cytotoxic activities on paper chromatograms. Use of this procedure as a disc-plate assay was illustrated and its application in the screening and fractionation of beers with carcinolytic activities *in vitro* was discussed.

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## Excretion of α-Ketoglutarate, Urea and Amino Acids by Normal and Muscular Dystrophic Mice.*† (25652)

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A preliminary chromatographic survey in this laboratory of urinary constituents of normal and muscular dystrophic strain 129 mice (1)‡ disclosed the presence of a substance which showed a reaction with semicarbazide reagent on chromatograms, and which appeared to be excreted in significantly larger quantities by dystrophic animals than by their littermate controls. The present report concerns identification of the unknown as α-ketoglutarate, further studies of excretion of this keto acid, and a simultaneous chromatographic investigation of amino acid and urea excretion. Amino acid excretion has been found altered or elevated in urines of other animals with various types of muscular dystrophy (2-7).

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‡ Obtained from Jackson Memorial Lab., Bar Harbor, Me.

*Methods. Collection and preservation of urine.* During urine collection periods, mice were housed in beakers with glass arms fused to the sides at a downward angle to serve as food containers. Access to food (ground Purina Chow) was given by means of holes in the sides of the beakers entering into the side arms. The mice were supported by wide mesh wire screens beneath which were placed at least 3 layers of glass beads pre-coated with thymol by immersion in a thymol-acetone solution followed by draining and drying. This arrangement tended to separate feces and scattered food from urine by allowing the latter to flow to the bottom of the beaker leaving the other materials trapped on top of the beads. Littermate pairs of mice, one dystrophic and one normal, were weighed and placed in the cages in the morning, and after 24 hours feces were removed and urine was collected with several washings. The combined washings were stirred, centrifuged, and supernatants were stored in glass vials at −10°C until analyzed. The urines of 5 pairs of mice were collected over various time intervals and analyzed for α-ketoglutarate, urea and amino