Tissue Culture. Bioassay Methods for Streptovitacin A.* (24768)

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A technic(1) for determination of *in vitro* carcinolytic activity of chemical compounds using a mammalian tissue culture system has been adapted to the assay of fermentation beers and extracts for cytotoxic activity. The assay of streptovitacin A, a new antitumor agent (2-7), in a tissue culture system is here described.

Materials and methods. Basic tissue culture technic is that of Eagle and Foley(1). Eagle's line of human epidermoid carcinoma was used for all assays. Stock cultures of KB cells† were maintained in Roux bottles containing approximately 100 ml of Eagle's basal medium‡(8) plus 10% horse serum. Cell population was determined by measuring the protein content(9). Stock bottles were planted with approximately 1 mg of cell protein, fed every 48 hours with 100 ml of fresh medium, and always fed 24 hours prior to subculturing or assay. For subculture, the medium was removed by decantation, the cells were scraped gently into 10 ml of fresh medium with a rubber policeman and were dispersed by rapid pipetting through a serological pipette. An aliquot was centrifuged, washed twice with Earle's salts (minus bicarbonate) (10) and the protein content determined. The cell suspension was diluted to give approximately 25 μg/ml of cellular protein, which was then gassed with 50% CO_2 in air. When 2 ml were dispensed into 16 x 150 mm test tubes, the pH equilibrated to approximately 7.2 at 37°C. Each tube was swirled thoroughly and incubated at a 5° angle at 37°C. At 24 hours the agent under test was introduced in fresh medium and appropriate media changes were made on the controls. At 72 hours, the medium (or medium plus agent) was renewed in all tubes, and at 96 hours protein content of the tubes was determined. For protein assay, the medium was removed by decantation, the tubes washed with two 5 ml portions of Earle's salts (minus bicarbonate) and the washed cells dissolved in biuret reagent. Protein content was determined by the Folin-Ciocalteau method(9). Results of tissue culture assay are expressed as ID_{50} (inhibitory dose for 50% inhibition) in μg/ml, calculated by plotting log of drug concentration *vs* per cent inhibition. Per cent inhibition was calculated from the following formula:

$$\% \text{ I} = \frac{\text{Final protein content in controls} - \text{final protein in tubes with agent}}{\text{Final protein in controls} - \text{initial protein in controls}} \times 100.$$

No corrections for medium blanks were made. Although minimum proliferation of KB cells in the absence of inhibitory agent of 6-fold was established as baseline for a successful control series, the cells generally increased from 7- to 8-fold in the 4 day test period.

Results. The log of drug dose was plotted against the % inhibition of KB cells in tissue culture, using crystalline streptovitacin A (Fig. 1). Determination of streptovitacin A activity on 4 different weeks can be plotted on a single, straight-line dose-response curve. Standard deviation per ID_{50} determination was 15% and per relative potency assay (comparing one material with another) was 8%, indicating that KB tissue culture assay for streptovitacin A is highly sensitive and reproducible. ID_{50} of crystalline streptovitacin A, calculated from the results presented above, was 0.055 μg/ml.

Activity of streptovitacin A in fermentation beers and extraction preparations was determined in an effort to follow the antitumor activity of crude materials. Figs. 2 and 3 show that several weeks' assays of crude strepto-

* The author wishes to acknowledge the valuable technical assistance of Mr. W. L. Lummis. Statistical analyses were performed by Dr. O. S. Carpenter, and Walker-256 assays by Dr. J. S. Evans. Extraction preparations were furnished by Dr. T. E. Eble, M. E. Bergy, and C. M. Large.

† Kindly supplied with much helpful advice by Drs. Harry Eagle and George Foley.

‡ Purchased from Microbiological Associates.

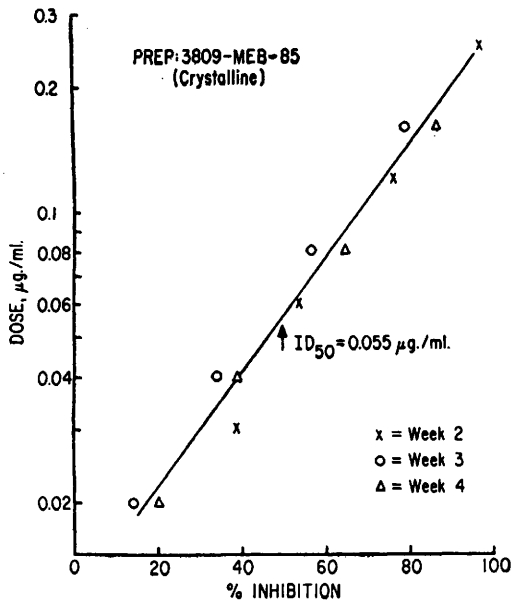

FIG. 1. Dose-response curve of crystalline streptovitacin A *vs* KB cells *in vitro*.

vitacin preparations can be plotted on a single dose-response curve, as in the case of crystalline streptovitacin A. Further, the slopes of dose-response curves for crude and crystalline streptovitacin preparations were nearly identical (Figs. 1-3). ID_{50} for preparation 3699-CML-70 was 0.85 µg/ml and for preparation 3587-TEE-98 it was 0.1 µg/ml.

Relative potencies of the above streptovitacin preparations were compared with relative activities obtained by an *in vivo* assay (Walker-256) and *in vitro* *S. pastorianus* assay (5) (Table I). The tissue culture assay was an accurate and reliable measure of the streptovitacin A content of crude preparations over a range of approximately 16 fold purification, and could have been used for isolation and purification of streptovitacin A, after cycloheximide had been removed.

The actinomycete which produces streptovitacin A also synthesizes cycloheximide. Activity of cycloheximide was determined by the assay procedure described above, and ID_{50} was found to be 0.1 µg/ml; approximately half that of streptovitacin A. Cycloheximide has been found to be less active than streptovitacin A *in vivo* (unpublished).

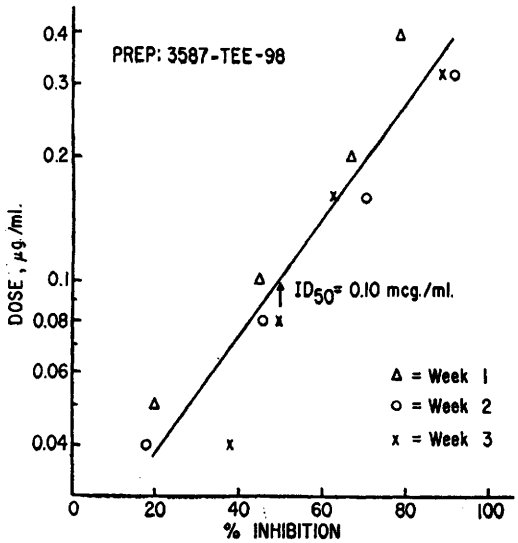

FIG. 2. Dose-response curve of partially purified preparation of streptovitacin A.

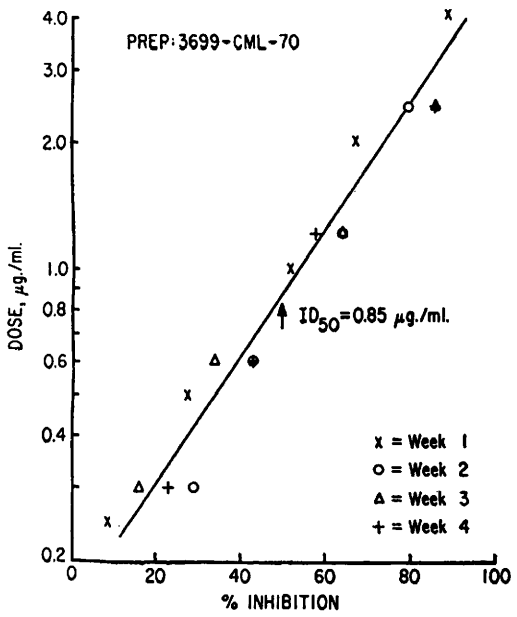

FIG. 3. Dose-response curve of crude streptovitacin A preparation.

Discussion. Results show that this tissue culture assay measured the activity of streptovitacin A and cycloheximide accurately both in crude preparations and in the crystalline state. Correlation between *in vivo* antitumor activity in rats and *in vitro* activity against KB cells in tissue culture was excellent Since the tissue culture assay measured the desired biological activity present in crude natural materials from which cycloheximide had been

TABLE I. Correlations between *in Vivo* and *in Vitro* Assays for Streptovitacin A.

Streptovitacin A preparation	Relative activities		
	Tissue culture	WAC*	*S. pastorianus*
1 (CML-70)	7	10	5
2 (TEE-98)	55	70	40
3 (crystalline)	100	100	100

* Walker-256, in rats (Evans, J. S., unpublished).

removed, it was a suitable assay for isolation and purification of streptovitacin A from fermentation beers. Total assay time was 5 days. This study emphasizes the rapidity, utility, sensitivity, and reproducibility of tissue culture assays for antitumor agents present in fermentation beers, provided a correlation exists between *in vivo* and *in vitro* activities.

Summary. A tissue culture assay employing Eagle's KB epidermoid carcinoma has been described which measured streptovitacin A concentrations between 0.02 and 0.25 μg/ml. Tissue culture assay measured streptovitacin A potency in crude beers and extraction preparations, in addition to crystalline material. Cycloheximide was also inhibitory to KB cells with an ID_{50} of 0.1 μg/ml. Standard error per ID_{50} determination was 15%, and per relative potency determination, 8%.

1. Eagle, H., Foley, G. E., *Am. J. Med.*, 1956, v21, 739.

2. Field, J. B., Costa, F., Boryczka, A., *Antibiotics Annual*, 1958,-1959, in press.

3. Field, J. B., Mirales, A., Pachl, H. R., Bascoy, L., Cano, L., Bullock, W. K., *ibid.*, 1958-1959,

4. Evans, J. S., Mengel, G., Ceru, J., Johnston, R., *ibid.*, 1958-1959,

5. Sokolski, W. T., Eilers, N. J., Savage, G. M., *ibid.*, 1958-1959,

6. Eble, T. E., Bergy, M. E., Large, C. M., Herr, R. R., Jackson, W. G., *ibid.*, 1958-1959,

7 Herr, R. R., *ibid.*, 1958-1959,

8. Eagle, H., *J. Exp. Med.*, 1955, v102, 595.

9. Oyama, V. I., Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 305.

10. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

Received February 9, 1959. P.S.E.B.M., 1959, v100.

Serum Lipid, Lipoprotein and Vascular Tissue Studies in Cholesterol-Fed Horse.* (24769)

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Shorland(1) noted that depot fats of various pasture-fed animals reflect the composition of dietary fats to different degrees. Body fats in horses and wild rabbits included 16 and 40% of linolenic acid respectively while fats from oxen, sheep and deer (ruminants) contained less than 1% (all animals having been at pasture in rye-grass, glycerides of which contained 63% of linolenic acid). This similarity of effect of dietary linolenate on fat depots of horses and rabbits prompted us to determine if cholesterol feeding would cause hypercholesterolemia and vascular lesions in the horse since this phenomenon is very striking in rabbits.

* This study was supported by grants from Nat. Heart Inst., U.S.P.H.S. (H-1889 & H-2528) and Oklahoma State Heart Assn.

Methods. A 1275 lb gelding approximately 12 years old and in apparent good health (cataract in one eye) was fed prairie hay and ½ U.S. peck of caramelized oats ("Carmol-oats") daily. U.S.P. cholesterol, 225 g daily, (estimated to constitute 1.7-2% of diet) was added to oats during 3 month period. The horse received no regular exercise. Blood was obtained by jugular puncture. Serum lipids and lipoproteins were measured before, during, and after cholesterol supplementation period. Total and free cholesterol were determined by the method of Sperry and Webb(2) and lipid phosphorus by modification (Hawk *et al.*,(3)) of the method of Youngburg and Youngburg. Lipoproteins were determined refractometrically at solvent density 1.21 g/ml after preparative centrifugation for 22 hours at 40,000