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Chemical Treatment of Tumors. III. Separation of Hemorrhage-Producing Fraction of *B. coli* Filtrate.

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Several investigators have found that hemorrhage could be produced in tumors by the administration of bacterial preparations and that the tumors subsequently receded in a significant proportion of cases. Confirmation of these findings has been reported.[1,2] However, the results obtained were not satisfactory inasmuch as small doses were often ineffective and large doses were toxic. The separation of the hemorrhage-producing agent from toxic and inert contaminants was therefore undertaken.

Little progress has hitherto been reported in this direction. The most recent relevant contribution is that of Apitz[3] who attempted to purify the "Shwartzman-active" substances present in *B. typhosus* and *B. paratyphosus* B preparations. Partial purification was effected, but Apitz found that the Shwartzman-active material so obtained was highly unstable; this instability halted further purification. It should be pointed out however that, in this study, Apitz was concerned solely with the Shwartzman phenomenon and reported no observations on the effect of this material on tumors.

The agent in *B. coli* filtrate responsible for hemorrhage production in mouse tumors was found to be stable in most instances, in this laboratory, even after a high degree of purity had been attained. The first steps in the separation of the active fraction were based upon the method devised by Felton, Kauffmann and Stahl[4] for the precipitation of the soluble specific polysaccharide from pneumococcus broth cultures.

The method used was, briefly, as follows:

The organisms were removed from week-old nutrient broth by centrifugation and by Berkefeld filtration. Phosphate was added to the filtrate; then the pH was brought to 9.5 by addition of lime water. The active material was carried down with the basic cal-

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1 Shear, M. J., and Andervont, H. B., Ann. Rep. of Surgeon General, Public Health Service of U. S., Washington, D. C., p. 25, 1932.

2 Shear, M. J., *Am. J. Cancer*, 1935, **25**, 66. (This paper also contains a review of the literature.)

3 Apitz, K., *J. Immunology*, 1935, **29**, 343.

4 Felton, L. D., Kauffmann, G. and Stahl, H. J., *J. Bact.*, 1935, **29**, 149.

cium phosphate precipitate, but a single precipitation did not always suffice to bring it down completely. In those cases in which precipitation failed to remove all activity from the supernatant, more phosphate was added and the pH again brought to 9.5 with lime water. This was repeated until all of the active material was removed from solution.

The calcium phosphate precipitates were combined, and dissolved in hydrochloric acid. Insoluble material was filtered off and discarded. Reprecipitation was then effected by the addition of sodium hydroxide to pH 9.5. The supernatant solution was inactive, as a rule, and was usually discarded. The precipitate was again dissolved in acid and again precipitated with sodium hydroxide. The object of these reprecipitations was to remove whatever protein had been carried down in the original precipitation.

The final calcium phosphate precipitate was then treated with acid and alcohol to remove the calcium. Enough hydrochloric acid was added to dissolve the precipitate and to bring the solution to pH 2. Insoluble matter was filtered off and discarded. The active material was precipitated from solution by addition of ethyl alcohol; the calcium remained in solution. Solution in acid and precipitation with alcohol was repeated until the precipitate was free from calcium. At times, anhydrous sodium acetate was added to the acid alcohol to promote complete precipitation of the active material.

This fraction was dissolved in water, neutralized with sodium hydroxide and evaporated to dryness. Finally, excess sodium chloride was removed by repeated treatment with methyl alcohol.

The purification was guided by testing the activity of each fraction on tumor-bearing mice. Since Andervont[5] had found that different strains of mice reacted somewhat differently to this agent, regularly reproducible results were insured by the employment of pure strain mice; most of the testing was done with Strain M mice. The fractionation was facilitated by the use of Andervont's skin-tumor technique[5] which provided a rapid, quantitative method of assaying activity. This made it possible to complete a determination of activity within 5 hours. For the most part, sarcoma 37 was employed as the test tumor although other transplanted sarcomas, as well as primary sarcomas induced by polycyclic hydrocarbons, were similarly affected by the agent.

By this procedure a water soluble fraction was obtained from *B. coli* filtrates which was highly potent in producing hemorrhage in mouse tumors. The minimum effective dose was 0.1 cc. of a

[5] Andervont, H. B., *Amer. J. Cancer* (In Press).

1:1,000 dilution of a stock solution which contained 22 mg. of the active fraction per cc. Hemorrhage was produced regularly by this dose, which contained 0.0022 mg. of active material.

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Chemical Treatment of Tumors. IV. Properties of Hemorrhage-Producing Fraction of *B. coli* Filtrate.

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The active fraction obtained from *B. coli* filtrate by the method described in the preceding paper was dissolved in water and evaporated to dryness *in vacuo*. A white residue was obtained, largely crystalline in nature. Strong heating produced some charring, but much of the crystalline residue was unaffected indicating that a considerable portion was inorganic in nature.

On repeated treatment of the active fraction with methyl alcohol, most of it (82%) went into solution. The methyl alcohol solution was evaporated to dryness and taken up in water, but most of it now failed to dissolve. However, addition of sodium hydroxide effected solution. Acidification with hydrochloric acid produced a heavy, flocculent precipitate which dissolved again on further addition of alkali. This solution was neutralized and tested on tumor-bearing mice; it was completely inactive as far as production of hemorrhage was concerned.

The methyl alcohol-insoluble portion (18%) dissolved readily in a small volume of water, with formation of a permanent foam. The presence of surface tension reducing material had been noted early in the purification, and its occurrence paralleled the presence of the active agent in the course of the fractionation. A stock solution was prepared containing 1 mg. per cc. of this fraction. This solution produced hemorrhage in mouse tumors in doses of 0.2 cc. of a 1:500 dilution, *i. e.*, the minimum hemorrhage-producing dose contained 0.0004 mg. of active material.

Upon evaporation to dryness *in vacuo* the active solution gave a residue which was largely non-crystalline. A comparatively small amount of crystalline material was present in which 3 crystal types were noted; the bulk of the residue consisted, however, of a film of non-crystalline glassy material.