Chronic Toxicity of Gossypol[1]

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The widespread publicity attained by gossypol as an appetite depressant, since the report (*3*) that highly purified preparations of gossypol act by delaying the passage of food from the stomach to the duodenum in the rat, has led to repeated requests for the use of this material for clinical trial in the treatment and control of obesity. Gossypol, a polyphenolic yellow pigment, is the principal component found in the pigment glands of cottonseed (*1*). Its acute oral toxicity and that of cottonseed pigment glands for rats, mice, rabbits, and guinea pigs have been reported (*2*).

To determine the effects of repeated oral doses of gossypol on the food intake and body weight, four young, litter-mate male dogs (5.0 to 5.4 kg) were given daily doses of 0, 50, 100 and 200 mg/kg body wt of the material, respectively, during three different experimental periods according to the following schedule: 55-day control period, 5-day experimental period (gossypol by capsule), 9-day control (rest) period, 5-day experimental period (gossypol by stomach tube), 9-day control period, 9-day experimental period (gossypol by stomach tube). From the first administration to the last, each of the three experimental dogs received a total of 19 daily doses of gossypol within a period of 37 days, which resulted in the death of all three dogs within 5 days after the last dose (one on the fourth and two on the fifth day).

The consistent effects of repeated doses of gossypol in the dogs were nausea, vomiting, diarrhea, anorexia, and marked weight loss. During the final period of gossypol administration the average food intake (dry basis) of each of the experimental dogs fell to 6.0, 0.4, and 3.0 g/kg body wt/day, respectively, while the control dog ate 28.0 g/kg body wt/day, and they lost 20, 26 and 25% of their body weight (the control dog lost 0.7%) within a period of 9 days. Post-mortem examination showed essentially the pathological findings reported for the rat, mouse, rabbit, and guinea pig (*2*), with marked lesions of focal necrosis involving the cecum, ileo-cecal valve, and adjacent portions of the large intestine. Further experiments are in progress.

It is suggested that the use of gossypol in human subjects be withheld until more data on its pharmacology and toxicology are available.

References

1. Boatner, C. H., Hall, C. M., O'Connor, R. T., and Castillon, L. E. *Bot. Gaz.*, 1947, **109**, 108–120.
2. Eagle, E., Castillon, L. E., Hall, C. M., and Boatner, C. H. *Arch. Biochem.*, 1948, **18**, 271–7.
3. Zucker, T. F., and Zucker, L. M. Abstracts of Papers. Amer. Chem. Soc., April, 1947, p. 47B.

[1] The samples of gossypol used in this study were supplied by the Southern Regional Research Laboratory, U.S.D.A., New Orleans, Louisiana, one of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration.

Application of Chromatography to the Separation of Subcellular, Enzymatically Active Granules

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It has recently been shown (*3*) that the chromatographic columnar adsorption method (*4, 6*) can be applied to the partial separation of biologically active particulates of virus dimensions from a chicken tumor extract. The present communication extends this chromatographic application to larger subcellular components, in the microscopically visible range, as found in pigmented mammalian tumors.

It has been found that melanized granules, varying in size from 0.2 to 0.6 μ or more, of the Cloudman S91 and Harding-Passey mouse melanomas, can be reversibly adsorbed on Celite columns (Fig. 1) and are thus subject to chromatographic manipulation. As a consequence, certain other constituents of the tumor homogenates employed as starting material can be readily separated from the granules, thereby providing a basis for noncentrifugal segregation of a substantial portion of the other tissue components. The particulate elements (*1, 2, 5*) separated by chromatography were found to possess high dopa oxidase and succinoxidase activities. As indicated by the

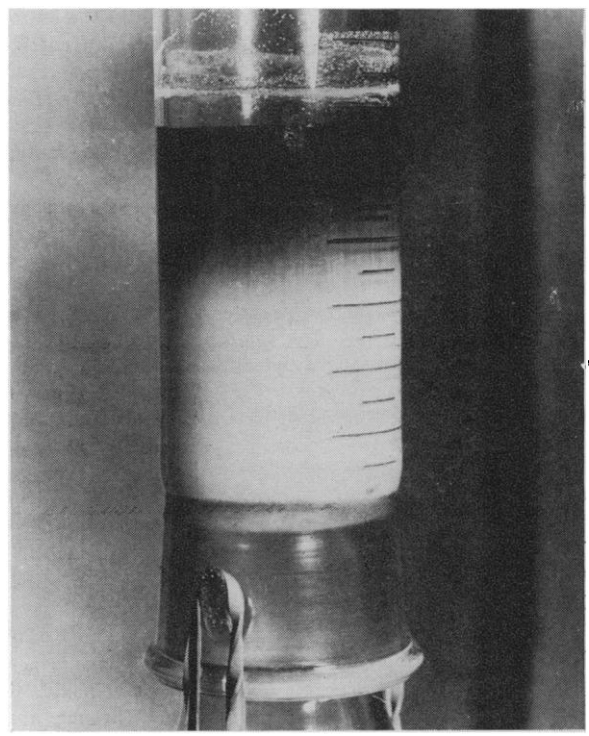

Fig. 1.—Adsorbed melanin granules on the developed chromatographic column prior to extrusion and segmentation.

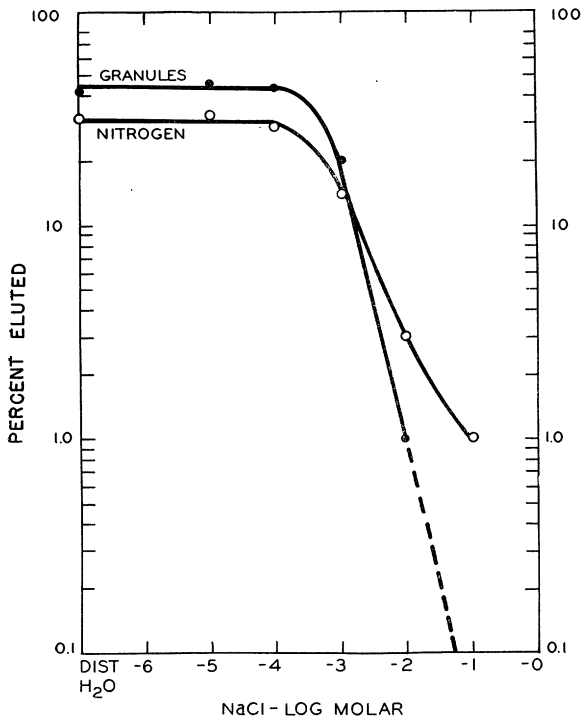

FIG. 2.—Effect of salt concentration on the elution of melanized granules and total nitrogen following the adsorption of equal quantities of a partially purified suspension on separate columns.

data below, granules so isolated possessed comparable or higher activities per unit of nitrogen than did the starting material, which had been partially purified by centrifugation, and considerably higher activities per unit of nitrogen than the starting extracts, where only preliminary centrifugal purification was employed.

In order to determine the adsorption and elution range of S91 melanin granules in this system, a curve was obtained by adsorbing from physiological saline equal quantities of granules (partially purified by centrifugation) on six identical 8 × 35 mm microcolumns. The columns were developed with 5 ml of additional physiological saline (0.154 M NaCl) and each column was then eluted with 10 ml of solution containing various concentrations of NaCl ranging from 10^{-1} to 10^{-5} M, and terminating with distilled water. Fig. 2 shows the influence of sodium chloride concentration on the elution of the melanin granules and total nitrogen, and indicates strong adsorption at 0.1 M, with continued influence through 0.001 M.

The percent granules eluted was estimated by measuring the optical density of the eluate at 650 mμ. A dilution curve of the centrifugally purified granules followed Beer's law, giving a straight line relationship with a zero intercept. Total nitrogen was determined by the Koch-McMeekin micro-Kjeldahl method,[1] and the enzymic activity of the granules was followed by their succinoxidase values determined manometrically in the Warburg apparatus. The pH of all solutions, including the tumor extracts, ranged from approximately 6.5 to 7.5. Within these limits, pH has little influence on adsorption or elution compared with the effect of salt concentration. In general, the adsorption and development procedures were carried out with cold solutions (2° to 10° C), while the elution was done with solutions at room temperature. The microcolumns were packed by introducing 5 ml of a 10% slurry of #503 Celite over a plug of absorbent cotton.

After establishing conditions for adsorption, development, and elution of the granules, the following experiment with a larger column was performed. Forty ml of a crude 20% S91 tumor extract[2] in saline was adsorbed on a dry Celite column 34 × 40 mm. A black zone of melanin granules formed in the upper part of the column and a pink zone containing hemoglobin and other tissue components developed immediately beneath the granules and migrated into the filtrate just behind a colorless zone of solvent. The column was developed with 40 ml of cold 0.154 M NaCl (0.9%), which was collected in the filtrate together with the pink fraction.

The moist column was extruded and cut into eight 5-mm segments, as illustrated in Fig. 3. In view of the adsorption-elution results shown in Fig. 2, each segment was separately eluted with distilled water, and the eluate tested for turbidity, succinoxidase activity, and nitrogen. The results are shown in Table 1 and Fig. 3. The data show that the granules were retained and subsequently eluted from the upper part of the column, while the preponderance of the starting nitrogen migrated into the percolate. This resulted in a more than 6-fold purification of the granules, as demonstrated by the relative increase in the succinoxidase $Q_{O_2}(N)$ value of fraction 2 compared with that of the starting material. The percolate (fraction 10), showed a corresponding decrease in activity, as did fractions 7 through 9. This adsorption behavior is similar to that found for the virus-like agent of chicken tumor I (*3*). The eluted particulate fraction 2 had a high population of melanized granules 0.3–0.6 μ in diameter, which were easily visible in the ordinary light and phase contrast microscopes.

If the above procedure is modified so that the elution is done by flowing chromatography rather than extrusion and segmentation, one black zone migrates down the column at a much faster rate than another, so that two distinct zones occur. While this indicates a chromatographic difference, or a change in a portion of the granules, the physiological or chemical differences of the two zones are yet to be studied. In experiments where this zone is left on the column it is expressed as elution inefficiency, as indicated in Fig. 2 and Table 1.

The association of the enzyme activity and the observed granules was attested by the fact that secondary

[1] Determined in Mr. Charles A. Kinser's laboratory, National Institutes of Health.

[2] This extract was prepared by grinding the tumor tissue at 1°–5° C with 0.9% NaCl to make a 20% homogenate. This was centrifuged for 3 min at approximately 1,000 x gravity, the supernatant was poured off, and its volume adjusted with saline to reestablish the original volume and yield a 20% saline extract. Such extracts contained most of the melanin granules, together with some nuclei and other coarse particulate matter.

centrifugation of such eluates at speeds just sufficient to sediment most of the particles yielded a fraction with succinoxidase $QO_2(N)$ values equal to or higher than the whole eluates. The same was also true for the dopa oxidase activity of the sedimented particles although, in contrast to succinoxidase, substantial quantities of dopa oxidase were also found in the saline and distilled water supernatants. Table 2 presents some of the dopa oxidase activities obtained when employing flowing chromatography. Further details concerning the different behavior of these two enzyme systems when manipulated chromatographically will be described elsewhere.

TABLE 1

RELATIVE DISTRIBUTION OF MELANIZED GRANULES, SUCCINOXIDASE ACTIVITY, AND TOTAL NITROGEN IN THE COLUMN ELUATES FOLLOWING THE ADSORPTION OF 40 ML OF A CLEARED TUMOR EXTRACT, AND DEVELOPMENT WITH AN EQUAL VOLUME OF 0.9% SALINE

Fraction	Oxygen consumption		Nitrogen		$QO_2(N)$		Optical density	
	µl/hr/ml	%	mg/ml	%	µl O_2/hr/mg N	%	D at 650 mµ	%
1. Starting extract*	16.7	100.0	1.48	100.0	11.3	100.0	1.40	100.0
2. Top 5 mm of column† ..	13.1	78.4	0.18	12.2	72.8	644.2	0.80	57.1
3. 2nd 5 mm of column ..	2.8	16.8	0.11	7.4	25.5	225.7	0.32	22.8
4. 3rd 5 mm of column ..	0.9	5.4	0.07	4.7	12.9	114.1	0.24	17.1
5. 4th 5 mm of column ..	1.5	9.0	0.07	4.7	21.4	189.4	0.20	14.3
6. 5th 5 mm of column ..	1.6	9.6	0.06	4.0	26.7	236.3	0.14	10.0
7. 6th 5 mm of column ..	0.2	1.2	0.052	3.5	3.8	33.6	0.09	6.4
8. 7th 5 mm of column ..	0.2	1.2	0.048	3.2	4.2	37.2	0.08	5.7
9. 8th 5 mm of column ..	0.3	1.8	0.062	4.2	4.8	42.5	0.07	5.0
10. Percolate‡	2.1	12.6	1.13	76.4	1.9	16.8	0.10	7.1

* See text footnote 2.
† All column segments eluted with 10 ml of solution, or ¼ the original volume of extract introduced on the column.
‡ Calculated at 40 ml starting volume.

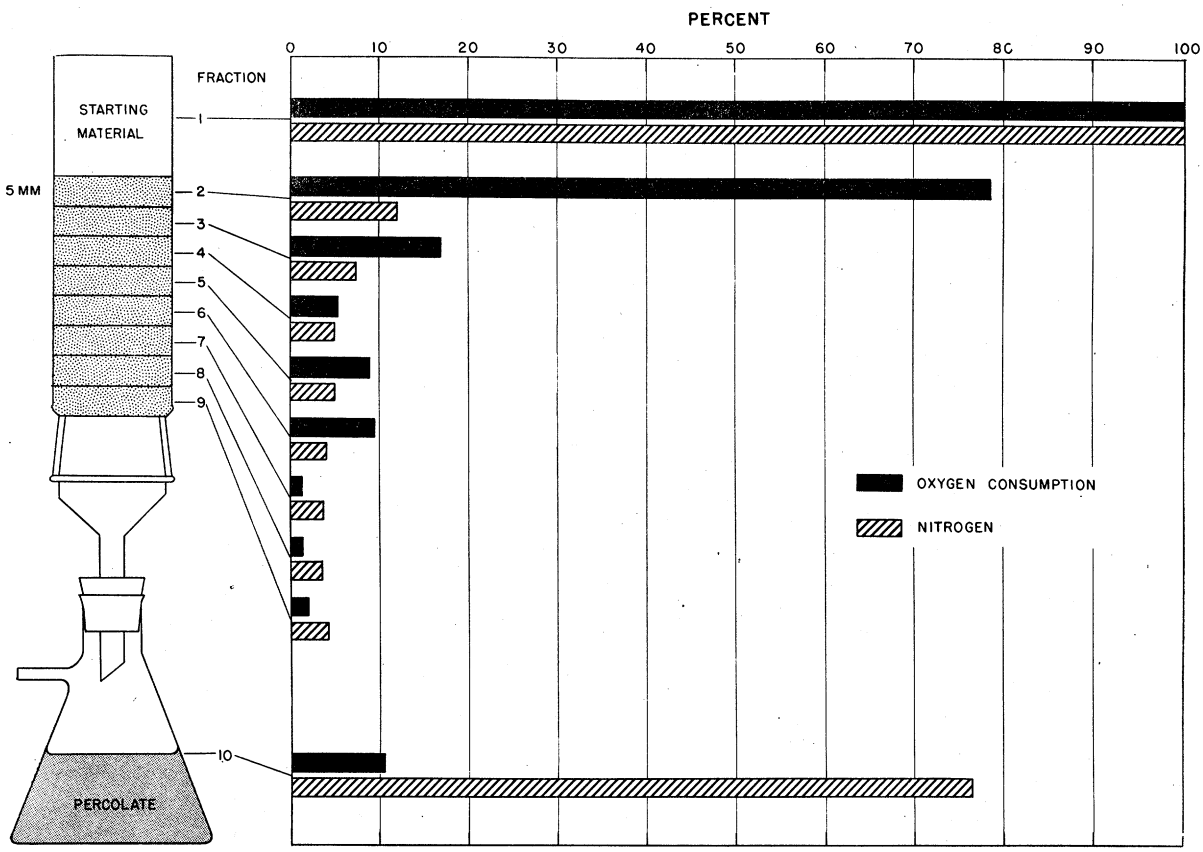

FIG. 3.—Distribution on the Celite column of melanized granules and other tumor components as indicated by the succinoxidase activity and the total nitrogen of the column eluates as compared with the percolate and the starting extract.

Preliminary studies have not shown any obvious staining or morphological differences in chromatographed granules as compared with smears or centrifuged preparations. There was, however, an apparent increase in uniformity of population. Fig. 4 shows an electron micrograph of Harding-Passey granules separated by flowing chromatography.[3]

The adaptation of chromatography to particulates ranging from virus to mitochondrial and bacterial size provides another method for separation and characterization of the particulate components of the cell. Since columnar chromatography presumably exploits the surface and molecular configurations of particles rather than their mass or specific gravity, the method may provide a new attack on the problem of separating morphologically similar units possessing different physical or chemical surfaces.

TABLE 2

RELATIVE DOPA OXIDASE ACTIVITIES PER UNIT OF NITROGEN IN S91 MELANOMA FRACTIONS OBTAINED BY PARTIAL CENTRIFUGAL PURIFICATION FOLLOWED BY CHROMATOGRAPHIC SEPARATION

Fraction	$QO_2(N)$ Value			
	Exp. 1	Exp. 2	Exp. 3	Mean
1. Starting extract*	23.5	13.1	14.2	16.9
2. Sedimented granules† ...	79.5	31.4	27.0	46.0
3. Column eluate‡	225.1	130.6	147.5	167.7
4. Sedimented granules from eluate§	372.4	185.4	78.1	212.0

* This extract prepared essentially as described in text footnote 2 except for being cleared twice at approximately 1,000 × gravity.

† This granule suspension consisted of all the material sedimented by subjecting the cleared starting extract to a force of approximately 4,000 × gravity for 10 min. The resulting pellet was resuspended to the original volume with 0.9% NaCl.

‡ The 10-fold increase in enzyme activity of this fraction compared with the starting extract has a $P < .001$ and is thus highly significant statistically. It is not known at present whether removal of an inhibitor is involved in this increase or not.

§ The granules in the column eluate were sedimented in the same way and simultaneously with those obtained in fraction 2 from the starting extract. This pellet was also resuspended to its starting volume with 0.9% NaCl.

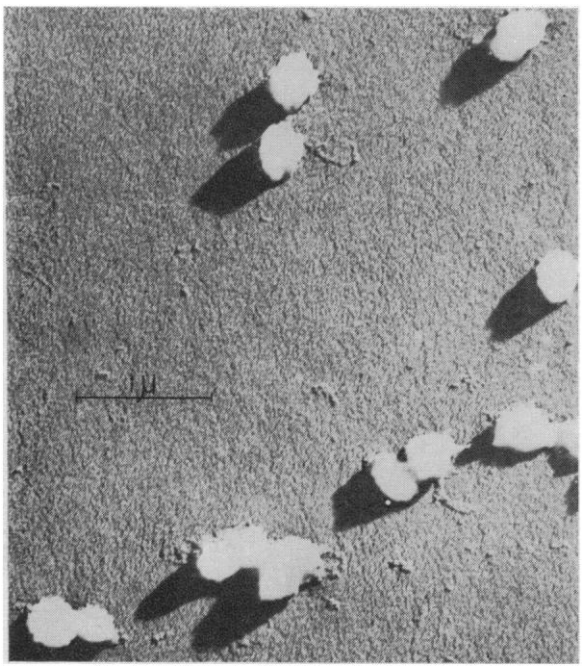

FIG. 4.—Electron micrograph of melanin granules from the Harding-Passey mouse melanoma obtained by chromatography. Gold shadowed at the approximate angle tangent 1/3. Magnification 8,000 ×. Enlarged to about 18,000 ×.

References

1. DUBUY, H., WOODS, M., BURK, D., and LACKEY, MARY. *J. Nat. Cancer Inst.* Feb. 1949.
2. MASON, HOWARD S., KAHLER, HERBERT, MACCARDLE, ROSS C., DALTON, ALBERT J. *Proc. Soc. exp. Biol. and Med.*, 1947, **66**, 421–431.
3. RILEY, VERNON T. *Science*, 1948, **107**, 573–575.
4. STRAIN, HAROLD H. *Chromatographic adsorption analysis.* New York: Interscience, 1945.
5. WOODS, M., DUBUY, H., BURK, D., and HESSELBACH, MARIE. *J. Nat. Cancer Inst.* Feb. 1949.
6. ZECHMEISTER, L., and CHOLNOKY, L. *Principles and practice of chromatography.* New York: John Wiley, 1943.

Lygus Bugs in Relation to the Occurrence of Embryoless Seeds in the Umbelliferae

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In fresh seeds of various members of the Umbelliferae family, quite often a considerable number—sometimes 50% or more—will not germinate, although they appear well filled and in good condition. Examination of many different lots of umbelliferous seeds (*3*) has shown that the fresh seeds which failed to germinate were without embryos while those containing mature embryos gave satisfactory germination. In these embryoless seeds the endosperm is present and from all appearances normal while the very small embryo which normally lies imbedded in the endosperm at one end of the seed is lacking. In no other family have such high percentages of embryoless seeds been reported; embryolessness, if present in other groups, is usually found in less than 1% of the seeds. The notoriously poor germination of umbelliferous seeds is evidenced by the rather low minimum germination standards set by the Federal government—55% for carrot and celery and 60% for parsley and parsnip. Evidence is presented in this paper that embryolessness occurs following the caging of Lygus bugs (*Lygus oblineatus* Say[1]

[1] Identified by Dr. Reece I. Sailer of U. S. National Museum.

[3] Electron microscopy by Dr. Herbert Kahler and Mr. Bolivar J. Lloyd, National Cancer Institute.