38, 1961 CC Orts

classification of material while filling or on account of differences between seed lots in the amount of lint, chaff, and debris. In some instances it may be desirable to determine the permeability of a specific seedpile. In this case the "Permeability Probe" devised during this investigation can be used to measure the permeability *in situ* quickly and easily.

Actual solution of Darcy's equation can be relatively simple, as shown in the example presented, or it can be quite difficult when a geometric "Shape Factor" needs to be obtained. It may be desirable to use an electrical analogue as described in this paper to determine the Shape Factor. An analogue also permits flow patterns to be established.

The analogue technique for solving Darcy's equation and the development of the Permeability Probe should be helpful in the design of aeration systems for many commodities other than cottonseed.

REFERENCES

1. Darcy, L., "Les Fontaines Publiques de la Ville de Dijon," Victor Delmont, Paris (1856).
2. Harris, W.B., "A Study of the Flow of Air Through Cottonseed Storage Units," graduate thesis, Texas A&M College, January, 1960.

[Received April 5, 1960]

NOTICE: This material may be protected by copyright

Studies of the Chick Edema Factor. II. Isolation of a Toxic Substance

ANDREW YARTZOFF, DAVID FIRESTONE, DANIEL BANES, WILLIAM HORWITZ, LEO FRIEDMAN, and STANLEY NESHEIM, Bureau of Biological and Physical Sciences, Food and Drug Administration, Department of Health, Education and Welfare, Washington, District of Columbia

A crystalline halogen containing material producing chick edema symptoms at 0.1 part per million in the diet has been isolated from a sample of triolein which was toxic to monkeys. This material is similar to that reported by Harman *et al.* (4) but differs somewhat in ultraviolet spectral properties.

IN A SYMPOSIUM on the chick edema disease in October, 1958, several laboratories (1,2,3) presented reports on their progress toward the isolation and elucidation of the toxic factor responsible for the occurrence of this unusual syndrome. It was established that the disease is caused by a toxic factor in the unsaponifiable fraction of a fatty by-product of industrial stearic and oleic acid manufacturing operations, and it was further suggested that the factor might possess a polynuclear or steroidal structure. A note, later published as an addendum to the contribution from this laboratory (2), reported that the toxic factor was associated with eluates from alumina and "silane-treated Celite" chromatographic columns which exhibited the ultraviolet absorption spectra of polysubstituted naphthalenes (λ_{Max} at 236 mμ, secondary λ_{Max} at 286 and 296 mμ). Neighboring cuts from these chromatograms showed the characteristic spectra of phenanthrene derivatives (λ_{Max} at 259, 282, 292, 300 mμ) and of simpler naphthalene derivatives (λ_{Max} 228 233 mμ, secondary λ_{Max} 270 280 mμ).

Subsequent purification of substances that had an absorption peak at 236 mμ demonstrated that they were not the most toxic fractions in our materials. Furthermore our computations showed that the toxic factor must be potent when present in the diet at levels of a fraction of one part per million. Recently Harman *et al.* (4) have reported the isolation of the chick edema factor in crystalline form from a feed-grade tallow. Their substance was toxic to chickens at 0.1 p.p.m. in the diet and had an ultraviolet absorption spectrum with a major peak at 244 mμ, a lesser peak at 312 mμ, and a shoulder at 238 mμ. A private communication from Tishler of the same laboratory (5) disclosed that the crystalline substance contains chlorine to the extent of about 47%.

Ames *et al.* (6) have observed the presence of the toxic factor in some commercial oleic acids. We have studied a sample of triolein which had been an ingredient in a series of dietary treatments involving changes in the level and types of fats to which a group of Cebus monkeys had been subjected.

The following summary of experimental results reltive to these monkeys was received from O. W. Portman and S. B. Andrus of the Department of Nutrition, Harvard School of Public Health.

Of a group of nine monkeys that received this triolein in their diets at a level of 25% by weight, one died at one month and four at three months. After three months on the triolein diet corn oil was substituted for the triolein. The other four monkeys died from three weeks to five months later even though triolein had been discontinued and replaced by corn oil. Of 14 monkeys in the colony that did not receive triolein but were supplied other fats and oils at 25% of the diet by weight, there was only one spontaneous death. Eight of the nine monkeys fed triolein were autopsied and showed the following findings: jaundice (4,8?); pancreatic atrophy and fibrosis (6); hemosiderosis (6); fatty liver (5); bile duct proliferation (3); extramedullary erythropoiesis (3); necrosis of liver (2); gross hemorrhage in gastrointestinal tract (2); and erythrocytophagocytosis (1). Several features including marked anemia in several instances suggested the possibility of a hemolytic process. Pancreatic changes were most pronounced in the two monkeys that survived longest (seven to nine months from the beginning of triolein feeding). The severity of the lesions in the pancreas was unrelated to that of the hepatic changes. With the exception of fatty changes in the liver, the above findings have not been reproduced in rats. These observations are from an experiment not designed to study a toxic principle, and it would be unwise to draw firm conclusions with respect to the toxicity of the triolein from these limited data.

The fact that, in our laboratory, marked symptoms of chick edema disease were produced by this sample of triolein suggests the possibility that the chick edema factor may have been responsible for the toxic effects noted in the triolein-fed monkeys.

We now wish to report the isolation of a highly toxic crystalline substance from this triolein and to describe its properties.

DSW 255058

STLCOPCB4060749

Experimental

The triolein sample was of excellent quality, with an unsaponifiable content of 0.87% and a steroidal hydrocarbon (1) absorbance of 0.07. Only 0.01% of oxirane oxygen (epoxide) was detected. Examination of the fatty acids as the ethyl esters by gas chromatography showed that oleic acid constituted 70.9% of the total acids with 3.6% linoleic, 0.3% linolenic, 13% palmitoleic, 9.4% palmitic and shorter-chain fatty acids, and 1.8% of a C_{17} fatty acid containing one double bond (by inference from relative retention time). Urea filtrate fatty acids were present to the extent of 4.73% (6).

When fed to chicks at a level of 15% in the diet, the triolein produced the symptoms of chick edema disease with a severity approximately equivalent to that observed with a diet containing the toxic fatty product used in our original studies (2) at the 5% level. The unsaponifiable fraction of the triolein was proportionately richer in the toxic factor than any other materials available to us.

The toxic factor in 17.6 kg. of triolein was concentrated by molecular distillation under pressures of 10–20 microns at temperatures up to 200°C. The distillate (289 g.), which contained all of the toxic factor, was saponified, and 166 g. of unsaponifiable material were recovered. The portion soluble in petroleum ether (155 g.), was chromatographed on 3 kg. of Fisher Alumina A 540 in a 4-ft. x 3⅛-in. column, using petroleum ether as eluent. Two-liter fractions were collected, and substances with the absorption spectra of naphthalene and phenanthrene derivatives were eluted in fractions 6–15. The bulk of the material containing cholestadiene and related hydrocarbons (129 g.) was eluted in the first three cuts, and the remainder was recovered by the use of more polar solvents. These foreruns and tailings were devoid of potency in the chick edema test.

Fractions 6 to 15 were combined (668 mg.) and chromatographed on 2,500 g. of the more retentive Merck Alumina No. 71707. No material was eluted with petroleum ether. Foreruns were eluted with 1–2% ethyl ether in petroleum ether, and fractions exhibiting the spectra of naphthalene and phenanthrene derivatives (239 mg.) were obtained when 5% ethyl ether in petroleum ether was employed as eluent. This material was again chromatographed on Merck alumina, using 250 g. in a 42 x 4.5-cm. tube. Several fractions which eluted with 5% ethyl ether in petroleum ether exhibited an absorption maximum near 245 mμ as well as the peaks previously observed at 234–236 mμ and 255–260 mμ. These fractions were combined, and the eluted material was partitioned on 5 g. of silane treated Celite, by reverse phase chromatography, using 4 ml. of iso-octane in the immobile phase, and 80% alcohol saturated with iso-octane as the mobile solvent.

Fractions exhibiting maximum absorption at 245 mμ, but not at 235 mμ or 255 mμ, were combined for two further chromatographic purifications on alumina. Merck alumina was deactivated with 3% its weight of water, and the ratios of alumina to sample and column size were 2,000:1 in a 25 x 1-cm. tube and 25,000:1 in a 25 x 2-cm. tube, respectively. Iso-octane, redistilled and chromatographed over silica gel, was employed as eluent, and fractions were again monitored by ultraviolet spectral absorbance. Fractions devoid of inflections at 235 and 255 mμ were combined and evaporated to dryness. The solid residue was dissolved in a small volume of boiling iso-octane and stored overnight in the refrigerator. White crystals weighing 2.61 mg. were obtained.

The ultraviolet absorption spectrum of the crystals dissolved in iso-octane was characterized by maxima at 245 mμ ($E^{1\%}_{1cm} = 718$) and 300 mμ ($E^{1\%}_{1cm} = 80$) and inflections at 240 mμ ($E^{1\%}_{1cm} = 655$) and 310 mμ ($E^{1\%}_{1cm} = 76$). The substance sublimed on the hot stage about 239°C. The infrared absorption spectrum of the substance, incorporated into a potassium bromide disc, showed evidence of aliphatic and aromatic linkages but no bands characteristic of oxygen or nitrogen functions.

The reported presence of chlorine in the toxic substance isolated by the Merck group (5) prompted a Beilstein test, which showed the presence of halogen. The presence of halogen in the crystalline material being reported here was confirmed by examination in a microcoulometric gas chromatograph.[1] (In this instrument the sample is fractionated by gas-liquid chromatography, and, as they are eluted from the column, the components are pyrolyzed at 800°C. under oxidizing conditions. The halogen acid formed from halogenated materials is titrated in a microcoulometer.)

In the chick bioassay the substance, when fed at *ca.* 1 p.p.m. in the diet, produced severe hydropericardium, hydroperitoneum, and liver damage, with death occurring within 12 days. At 0.1 p.p.m. in the diet marked hydropericardium was evident at autopsy after a three-week feeding period.

The isolation of the toxic substance had been complicated by the presence of another material with an absorption maximum at 248 mμ. This second substance was isolated by the same techniques of chromatography, monitored by ultraviolet spectrophotometry, and a yield of white crystals was obtained. The ultraviolet spectrum of this material was identical with that of the toxic substance except that it was shifted 3 mμ so that the major absorption peak was at 248 mμ. It behaved similarly to the toxic substance in the microcoulometric gas chromatograph, showing a similar retention time and a similar halogen content. However it was completely inactive in the chick edema test when fed at 1 p.p.m. in the diet.

In order to obtain additional crystalline material the portion remaining from nondestructive testing, fortified with the adjacent fractions from the final chromatography (of high potency, judging from the ultraviolet spectrum), was further chromatographed according to the previous isolation scheme. Small quantities of phenanthrene derivatives were separated with a consequent diminution in the ultraviolet absorption in the 250–300 mμ region. Furthermore, although there was no indication of nonhomogeneity by paper chromatography, the ratio of absorbances of the shoulder at 240 mμ to the peak at 245 varied from fraction to fraction, suggesting the presence of an additional component.

The extinction values reported above may be in error because of inaccuracies in weighing on account of the difficulties in handling the small amount of material involved. There is no doubt however of the validity of the relative absorbancies at various wavelengths. The spectrum of the substance reported here

[1] Dohrmann Manufacturing Company, Palo Alto, Calif.

DSW 255059

STLCOPCB4060750

differs somewhat from that reported by Harman *et al.* (4), particularly in the ratio of the absorbance at the maximum (244 or 245 mμ) to the absorbance at the inflection at 238 or 240 mμ.

Discussion

The toxic substance which we have isolated from triolein resembles that recovered by Harman *et al.* (4) from animal feed tallows. However the divergences in their properties suggest either that we are dealing with two different but closely related compounds, or that one or both of the preparations is still a mixture of related compounds despite the fact that only a single spot could be obtained on paper chromatography in a number of solvent systems. In view of the manifest difficulties involved in isolating a pure compound in minute quantities from a myriad of substances with similar properties it would be hazardous, as Harman warns, to infer chemical structures from spectral data. Nevertheless it should be pointed out that the spectra obtained by us and by Harman *et al.* are strongly reminiscent of those exhibited by highly substituted naphthalenes (7). Furthermore the toxic factor occurs in association with a bewildering array of aromatic naphthalene and phenanthrene derivatives, as we have previously noted (2). The detection of chlorine in large proportions in a toxic preparation, and the ultraviolet spectrum observed, suggest a possible relationship with chlorinated naphthalenes. Pentachloronaphthalene possesses an absorption maximum at 243 mμ and a secondary maximum at 312 mμ (8), and it has been shown to cause hyperkeratosis in cattle (9) and several other species of animals including chickens (10). Other chlorinated naphthalenes also are toxic (11).

The possibility that the chick edema factor is a chlorinated naphthalene derivative cannot be ignored. Samples of tetrachloronaphthalene and hexachloronaphthalene, kindly provided by Engel and Bell of the Virginia Polytechnic Institute, who had demonstrated that these compounds could produce hyperkeratosis in cattle, were without effect in the chick edema test. Furthermore these compounds, despite the similarity of their ultraviolet spectra and their chromatographic behavior to the toxic substance, showed considerable difference in the microcoulometric gas chromatograph. For instance, the chlorinated pesticides, aldrin and heptachlor, showed retention times of 10 min., tetrachloronaphthelene 9 min., and hexachloronaphthalene 14 min., whereas the toxic substance, as well as its inactive analogue with the absorption maximum at 248 mμ, had retention times of 37–38 min. It is tempting to speculate that the greater retention-time of the toxic material is related to a greater molecular weight or to a substituent conferring different solubility and polarity properties.

We are continuing our studies toward the isolation of the toxic factor. It is necessary that the chemical nature of this substance be elucidated to make possible a rapid chemical test for its detection, to clarify its origin, to verify the suggestion of its severe toxicity to primates, and to study its action in other species.

Acknowledgment

Numerous individuals in addition to those mentioned in the text have contributed directly or indirectly to this project. We are particularly grateful to O.L. Kline for his sustained interest, encouragement, and helpfulness in the course of these investigations; to Benjamin Webb, who conducted the extensive molecular distillations; to Donald F. Flick and Linda Gallo for the chick bioassays; and to Raymond J. Gajan for the microcoulometric gas chromatography. The cooperation of the various commercial laboratories that were also working on this problem, in discussing their work with us, is also greatly appreciated.

REFERENCES

1. Brew, W.B., Dore, J.B., Benedict, J.H., Porter, G.C., and Sipas, E., J. Assoc. Offic. Agr. Chemists, *42*, 120–128 (1959).
2. Friedman, L., Firestone, D., Horwitz, W., Banes, D., Anstead, M., and Shue, G., *ibid.*, 129–140.
3. Wooten, J.C., and Alexander, J.C., *ibid.*, 141–148.
4. Harman, R.E., Davis, G.E., Ott, W.H., Brink, N.G., and Kuehl, F.A., J. Am. Chem. Soc., *82*, 2078–2079 (1960).
5. Tishler, M., Merck and Company, private communication, July 19, 1960.
6. Firestone, D., Nesheim, S., and Horwitz, W., J. Assoc. Offic. Agr. Chemists, in press.
7. Abadir, B.J., Cook, J.W., and Gibson, D.T., J. Chem. Soc., 1953, 8.
Mosby, W.L., J. Am. Chem. Soc., *75*, 3348–3349 (1953).
8. Blickenstaff, R.T., and Callen, J.E., Anal. Chem., *26*, 1586–1589 (1954).
9. Sikes, D., and Bridges, M.E., Science, *116*, 506–507 (1952).
10. Köhler, H., Archiv. Experimentelle Veterinarmedizin, *8*, 163–198 (1954).
11. Bell, W.B., Vet. Med., *48*, 135–140 (1953).

[Received October 17, 1960]

Determination of the Glyceride Structure of Fats[1,2]

C.G. YOUNGS, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada

A method has been described for the quantitative determination of the following six glyceride types in fats: SSS, SSU, SUS, SUU, USU, and UUU. The method involved a quantitative oxidation of the unsaturated acids in the whole fat to the corresponding dicarboxylic acids. The oxidized fat was separated on a liquid-liquid partition column into two fractions, the first containing glycerides having no dicarboxylic acid or one dicarboxylic acid and the second containing glycerides with two or three dicarboxylic acids. Analysis of these fractions by gas chromatography coupled with lipase hydrolysis allowed the calculation of the proportions of the above six glyceride types.

The oxidation, fractionation, lipase hydrolysis, gas chromatographic analysis, and the over-all method were checked on natural fats and mixtures of synthetic glycerides. The final glyceride composition appeared to be reliable to within plus or minus 2 unit per cent.

Analyses are given for five natural fats. The compositions found agree very well with those calculated by a distribution theory recently proposed by Vander Wal.

[1] Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
[2] Issued as N.R.C. No. 6161.

DSW 255060

STLCOPCB4060751