Journal of Chromatography, 86 (1973) 107–116

CHROM. 6962

DETECTION AND QUANTITATIVE MEASUREMENT OF FECAL WATER POLLUTION USING A SOLID-INJECTION GAS CHROMATOGRAPHIC TECHNIQUE AND FECAL STEROIDS AS A CHEMICAL INDEX

J. DOUGAN and L. TAN*

Department of Biochemistry, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec (Canada)

(Received April 18th, 1973)

SUMMARY

A new method for the gas chromatographic identification and quantitation of fecal steroids by the solid-injection technique is described. Separation of these steroids was achieved by use of a combination column consisting of OV-1 and OV-210 coated phases. Coprostanone is proposed as the most suitable chemical index for measuring fecal water pollution. With our method, it is possible to measure this compound at a contamination level in the 400 picogram per milliliter range. No simple relationship seems to exist between coprostanone content of polluted water samples and classical coliform counts.

INTRODUCTION

The discovery of 5β-sterol formation by stereospecific reduction of cholesterol by the intestinal flora in mammals[1–8] has led to the suggestion of the potential use of coprostanol (5β-cholestan-3β-ol) as a molecular tracer in the gas chromatographic detection of fecal contamination of natural waters[9,10]. More recently, the single combined packed column described previously[11] allowed the separation of the formerly coincident cholesterol and coprostanol peaks. The latter paper also suggested for the first time the use of coprostanone (5β-cholestan-3-one) as a well-resolved steroid chemical index of fecal contamination. The distinct resolution of these three fecal steroids (cholesterol, coprostanol, coprostanone), if coupled to a suitable quantitative analysis, thus promised to provide a practical method for the chemical measurement of fecal water pollution.

Initially, problems arose in the development of an acceptable analytical procedure for the quantitation of the above mentioned steroids. Firstly, other lipid contaminants present in natural water samples, chiefly esters, fatty acids and other volatile material, made the gas chromatograms difficult to quantitate by conventional techniques, due to excessive background peaks. Secondly, the use of solvents for injection at the high detector sensitivity settings required to measure the steroids quantitatively in the nanogram range resulted in extensive solvent fronts which often

* Author to whom inquiries should be addressed.

buried the steroid peaks of interest. A different procedure, involving the prepurification of the crude extracts by thin-layer chromatography, combined with a solid-injection technique, has enabled us to eliminate these two major obstacles. The detection and subsequent quantitative measurement of fecal steroids present in actual water samples by this new analytical method is the subject of this paper.

MATERIALS AND METHODS

Materials

Reference steroids were obtained from Steraloids, Inc. (Pawling, N.J., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.). All solvents used were reagent grade, and were redistilled prior to use. Thin-layer chromatography was performed on glass plates coated with silica gel PF_{254} (E. Merck, Darmstadt, G.F.R.) according to methods described earlier[11,12]. The gas chromatograph used was a Hewlett-Packard Model 7610A instrument (Hewlett-Packard, Avondale, Pa., U.S.A.), equipped with dual hydrogen flame ionization detectors, and operated under the following conditions: oven temperature 210°, injection port and detector temperatures 265°, nitrogen carrier gas flow 55 ml/min at 40 lb./sq. in. A single, combined packed column, composed of 3% OV-1 and 3% OV-210, coated on 100–120 mesh Gas-Chrom Q, which were added simultaneously to opposite ends of a 180 cm × 2 mm I.D. U-type glass column, was used. For micro-measurement of liquid samples, a Glenco 10-μl syringe was used. The solids injector syringe employed was the Solids Injector SI-1 (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia), equipped with a stainless-steel spiral injection tip.

Methods

I. Determination of column properties

Column efficiencies for cholesterol, coprostanol, coprostanone and cholestenone, as well as their retention times relative to 5α-cholestane were calculated.

II. Extraction of fecal steroids from surface waters, and coliform countings

Water samples, 12 l in size, were taken at various time intervals in the year from a number of lakes and rivers in the Eastern Townships region of Quebec, Canada. The samples were collected in polyethylene "jerry-cans" and were transferred to 20-l glass bottles, usually on the day of collection. Simultaneously, 200–500 ml of water samples were taken in sterile, screw-capped erlenmeyer flasks for the purpose of coliform counting, which was performed within a few hours of the collection.

Microbiological analysis. Coliform counts were made at dilutions of 1, 10 and 100 ml of sample by the membrane filter technique[13], using M-Coliform broth, incubated at 44° for 24 h. Only those colonies with a greenish-gold sheen and dark centres were counted.

Chemical work-up. Following the addition of 2 l of re-distilled hexane, the 12-l water samples were stirred to a deep vortex for 15 min, using a motor-driven stainless-steel shaft and propeller. After separation of the two liquid phases, the hexane fraction was siphoned off, dried over anhydrous sodium sulfate, filtered through a cotton plug, and the total volume of hexane retrieved noted. The organic extract was evaporated under vacuum using a Buchi "Rotavapor", and the semi-liquid residue

transferred with several washings to a small capped vial, using acetone. The latter solvent was then removed under a gentle stream of air, and the product thus obtained stored at 4° until further use.

III. Qualitative gas–liquid chromatographic analysis

Approximately 5 ml of redistilled dioxane were added to the residue obtained from the water extraction. Two microliters of this solution were applied to the spiral part of the solids injector syringe, using a Glenco microliter syringe, and the solvent was allowed to evaporate at *ca.* 100°. The solid sample was then injected into the combined OV-1/OV-210 column at an attenuation of 10×2 or 10×4. The gas chromatograms obtained were then scrutinized for the presence of the fecal steroids of interest. The remaining dioxane solution was air-dried and set aside for further purification by thin-layer chromatography.

IV. Preparative thin-layer chromatography of extracts

A preparative 20×20 cm glass plate coated with 1–2 mm silica gel was heated at 110° for 1 h. The plate was then divided into four narrow strips and one broad band. Reference cholesterol, coprostanol, coprostanone and cholestenone were spotted on the four narrow strips, while the water extract, dissolved in benzene, was applied to the broad band, either manually or using a motor-driven mechanical streaker (Rodder Instruments, Palo Alto, Calif., U.S.A.). The plate was developed in chloroform–ether (9:1) and allowed to dry. The four narrow strips were sprayed with 50% sulfuric acid, and heated on a hotplate until appearance of the steroid chromogens. The plate was then examined under an UV light source at 254 and 366 nm. Following comparison with the R_F values of the steroid standards the appropriate portions of the broad sample band were scraped off and transferred into 50-ml erlenmeyer flasks. The silica gel was extracted with 5–20 ml of a mixture of chloroform–methanol (9:1) and after settling, the clear liquid was sucked off with a pipette and filtered through a Whatman No. 4 filter paper. This elution was repeated three times. Finally, the remaining suspension was transferred to the filter paper and thoroughly rinsed with solvent. The filtrates were transferred to a 20-ml test tube, and the solvent removed with a gentle stream of air. The residue was re-dissolved in a small amount of solvent and transferred, using a Swinney filter syringe, to a capped vial. After evaporation of the solvent, the vial was stored at 4°.

V. Preparation of standard curves

Standard solutions of coprostanol (5.20 mg/100 ml) and coprostanone (5.58 mg/100 ml) in dioxane were prepared. An internal standard (I.S.) had to be chosen which (i) would be well-resolved from the fecal steroid peaks, (ii) would not show any tailing, and (iii) is not normally present in natural water samples. Cholestenone (4-cholesten-3-one) was finally selected for meeting all the above requirements. A standard solution of cholestenone (10.66 mg/100 ml) in dioxane was thus prepared. Varying amounts of reference coprostanol or coprostanone were applied to the spiral of the solid syringe, together with a measured amount of the I.S. These co-injections were repeated at least six times. To determine the ratio of fecal steroid peak weight to I.S. peak weight for each injection, the gas chromatograms obtained were redrawn on high-quality tracing paper, and the peak areas cut and weighed. A curve was plotted from the

average values, using ratio of weights on one axis and nanograms of fecal steroid injected on the other. Using the method of least squares, a straight line was then fitted.

VI. Quantitative gas–liquid chromatographic analysis of water sample extracts

To the residue obtained as described previously under *IV*, 0.50 ml of dioxane was added to the vial using a micropipette. With a microliter syringe 1.0 μl of this solution was withdrawn and applied to the solid syringe, which was mounted in a specially constructed heating block. Exactly 1 μl of I.S. standard solution (corresponding to 106.6 ng of cholestenone) was also applied on the spiral of the solid syringe. After the solvent had evaporated, the solid syringe was inserted into the injection port of the gas chromatograph and held there for 6 sec before removal, to allow complete evaporation of the applied materials. The gas chromatograms were then traced, the peak areas cut and weighed as described before under *V*, and the weight ratios determined. From the weight ratios thus obtained, and by using the appropriate standard curve, the concentrations of the fecal steroids extracted were calculated.

VII. Determination of the efficiency of the procedure

To a 12-l volume of distilled water were added 10 mg each of coprostanol, coprostanone, cholesterol and cholestenone, dissolved in dioxane. The mixture was thoroughly mixed for 30 min with power stirring. The steroids were then extracted with 2 l of hexane, applied to a thin-layer plate, retrieved by elution, and quantitated by GLC as described previously. From the amounts of steroids retrieved, the efficiency of the entire procedure was determined.

RESULTS AND DISCUSSION

Separation of cholesterol, coprostanol and coprostanone is easily achieved with our combination column, while the I.S., cholestenone, exhibits a retention time which does not interfere with those of the fecal steroids (see Fig. 1). It should be noted that GLC analysis on a column, packed with a non-polar or polar phase alone, would result in coalescent peaks of the pairs cholesterol–coprostanone and cholesterol–coprostanol[11]. Table I shows the efficiency of the combination column towards the four steroids studied and also the retention times. The heating block which we have used for mounting the solid syringe is shown in Fig. 2. It consists of an aluminium plate with appropriate holes drilled. The whole plate was kept at a pre-determined temperature on a thermostatically controlled hotplate at just below 100°. At this temperature, rapid evaporation of the minute amounts of solvents was ensured, while preventing thermal decomposition of the steroids. The standard curves which we have obtained for the quantitative measurements of coprostanol and coprostanone are shown in Figs. 3 and 4, respectively.

A water sample, taken from a lake in the Sherbrooke region, was next analyzed by gas chromatography using the solid-injection technique (Fig. 5). This gas chromatogram was obtained after prior purification of the water extract by preparative TLC. Most of the more volatile lipid material was hence removed and the steroid peaks of interest can clearly be distinguished from the remaining background peaks.

In Table II, we have summarized our findings of water samples, collected from

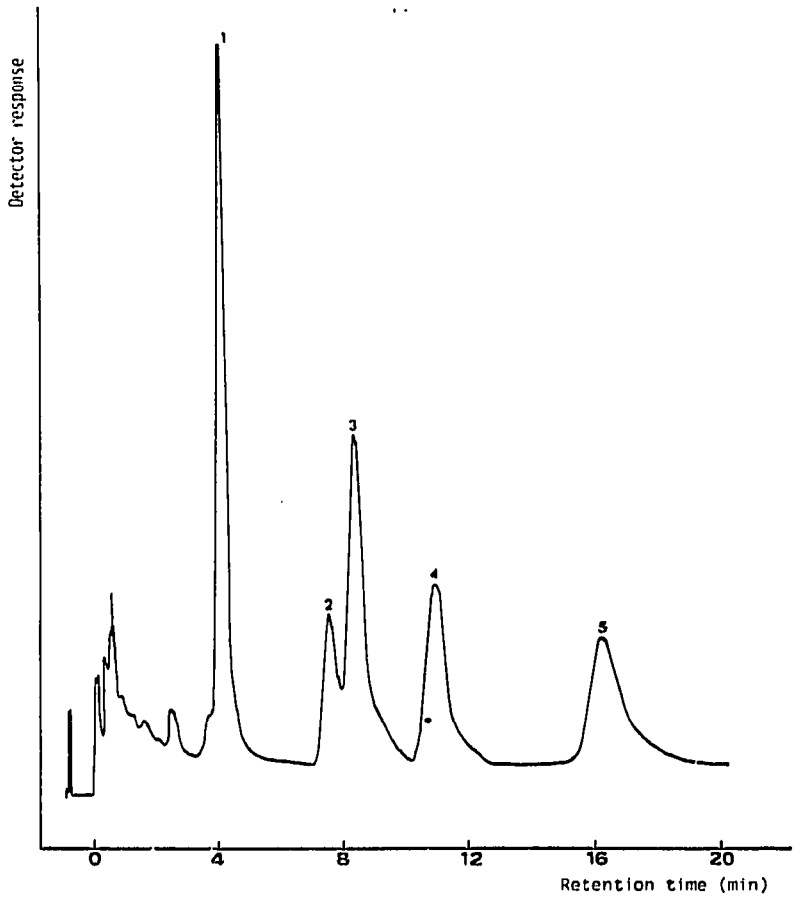

Fig. 1. Gas chromatogram obtained via the solid-injection technique of reference steroids on a 180 cm × 2 mm I.D., 3% OV-1/OV-210 combination column operated under the following conditions: oven temp. 210°, injection port temp. 265°, detector temp. 265°. Nitrogen carrier gas flow 55 ml/min; attenuation 10×8. 1 = 5α-Cholestane; 2 = coprostanol; 3 = cholesterol; 4 = coprostanone; 5 = cholestenone.

TABLE I

SUMMARY OF CHROMATOGRAPHIC DATA

Compound	*TLC*	*GLC***	
	*R_F**	*RRT*	*Theoretical plates*
5α-Cholestane	—	1.00	420
Coprostanol	0.45	1.94	1024
Cholesterol	0.31	2.09	824
Coprostanone	0.76	2.77	687
Cholestenone	0.60	4.18	890

* Solvent system: chloroform–ether (9:1).

** On 3% OV-1/OV-210 and oven temperature of 210°.

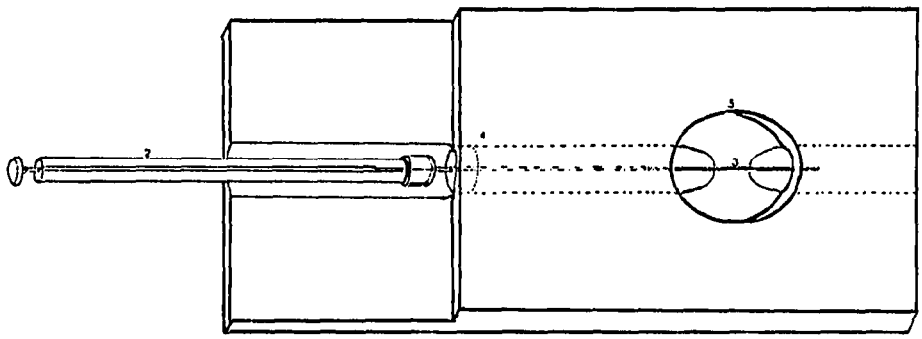

Fig. 2. Upside view of aluminium heating block for preparation of solid injection samples. 1 = Solid aluminium heating block; 2 = solid injection syringe; 3 = spiral; 4 = rubber septum; 5 = access port.

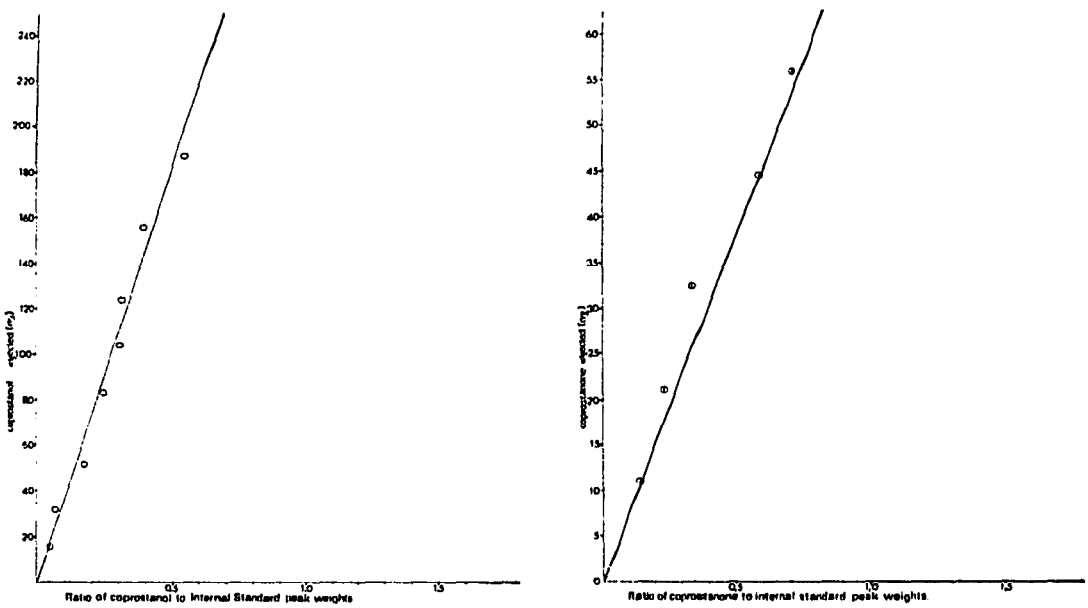

Fig. 3. Standard curve of coprostanol obtained with the solid-injection technique. Varying amounts of coprostanol were co-injected with 106.6 ng of cholestenone (I.S.) and the peak area ratios determined by cutting and weighing.

Fig. 4. Standard curve of coprostanone *versus* cholestenone as an internal standard (106.6 ng), obtained via the solid-injection technique.

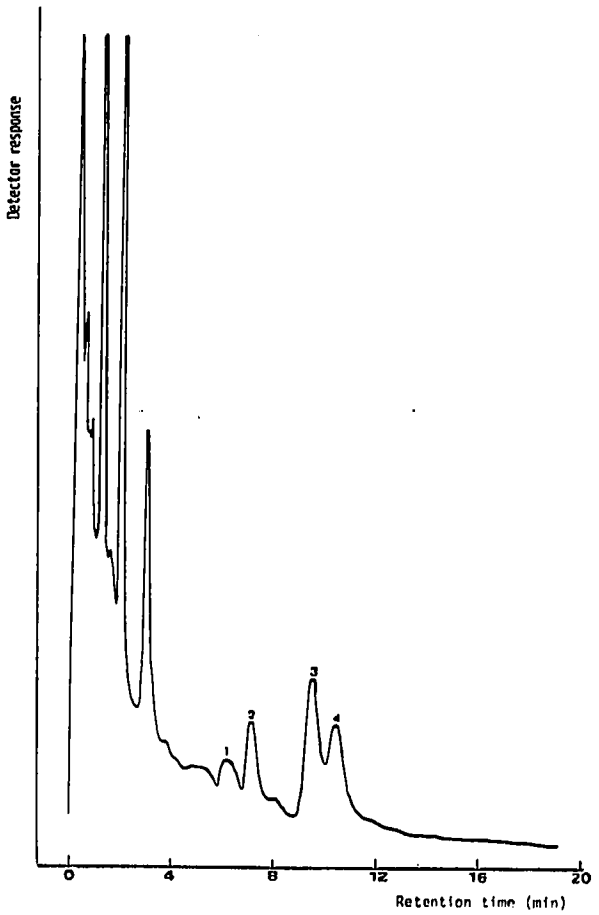

Fig. 5. Gas chromatogram of an actual water sample, prepurified by thick-layer chromatography, and obtained via solid injection. Nitrogen carrier gas flow: 55 ml/min; attenuation 10 × 2. Other conditions: same as in Fig. 1. 1 = Coprostanol; 2 = cholesterol; 3 = coprostanone; 4 = unknown contaminant.

TABLE II

COMBINED MICROBIOLOGICAL-CHEMICAL ANALYSIS OF WATER SAMPLES

Sample No.	*Coliform per 100 ml*	*Coprostanol in ng per 100 ml*	*Coprostanone in ng per 100 ml*
1	330 ± 7	193 ± 9	221 ± 7
2	80 ± 2	2206 ± 103	255 ± 2
3	440 ± 9	–	155 ± 2
4	86 ± 2	–	1002 ± 24
5	480 ± 9	–	111 ± 9
6	38 ± 2	352 ± 7	43 ± 2

different places in the Eastern Townships region of Quebec. In all these samples, cholesterol seems to be always present, as is coprostanone. On the other hand, coprostanol is not always detectable. We assume that when this compound is accumulating for longer periods in natural waters, it can undergo autoxidation to yield the more stable coprostanone. It cannot be excluded, however, that when coprostanol is present in much smaller amounts than cholesterol, it is not detectable in the gas chromatogram, because its response would be buried within the major cholesterol peak. In any case, coprostanone is clearly the much better index: it is widely separated in GLC from accompanying cholesterol, it does not exhibit peak tailing, and it is chemically more inert in natural waters than coprostanol.

As can be deduced from Table II, there appears to be no direct relationship between the number of coliform bacteria, and the concentration of coprostanol or coprostanone in fecally polluted waters. Thus, in samples Nos. 2 and 4, where the coliform number is low, we have a high concentration of coprostanol and coprostanone, respectively. On the other hand, in samples Nos. 3, 4, and 5, where coprostanol could not be detected, there is clear evidence for the presence of its oxidation product coprostanone.

Usually, public health services confine the analysis of fecal contamination of water to microbiological methods only. The coliform bacteria[14–17] serve as indicator organisms, their populations reflecting the presence or absence of other pathogenic bacteria. In most cases, the identification of specific coliform strains of fecal origin is a long and tedious process, which is prone to inaccuracies, as emphasized in the monograph of Geldreich[18]. The survival of bacteria in water is controlled to a large extent by extremely variable environmental factors such as pH, osmotic and hydrostatic pressure, temperature, oxygen concentration, light intensity, chemical contaminants, etc. Protozoans and bacteriophages are known to be destructive to bacteria[19–21]. Also, non-mammalian sources of coliforms have been noted which in certain situations could account for many of the coliforms counted. It is therefore not surprising that no single medium has as yet been discovered which permits the isolation of coliforms of fecal origin exclusively[18].

We have therefore deemed it necessary to look for a complementary method for the detection and more accurate measurement of fecal contamination. Proper selection of a compound of animal and fecal origin, which is inert under the ecological conditions normally present in natural waters, appears to be the answer to our needs. Studies by steroid biochemists have indicated that the intestinal microflora of higher mammals produce significant quantities of the sterol coprostanol. This compound, in turn, may undergo further oxidation to coprostanone, both within and exterior to the body. The formation of these compounds is impossible without the action of intestinal microflora, as demonstrated by Gustafsson *et al.*[8] in their studies with germ-free rats. We have, therefore, in these 5β-cholestane metabolites a chemical index or tracer molecule of fecal pollution. These compounds have the additional significance of being amenable to precise quantitative determinations, in contrast to bacterial counts, which are at best crude approximations of the degree of fecal contamination.

The water samples analyzed (Table II) bear out these opinions. Samples Nos. 2, 3 and 6 were taken from the same location, but at different periods in the year. Sample No. 2 was collected early in June, No. 3 in midsummer, and No. 6 in

December. The bacterial counts reflect very well the changes due to the season. On the other hand, the coprostanone measurements appear to be less influenced by the seasonal changes.

In quantitative studies, we recommend the use of coprostanone rather than coprostanol. Coprostanone is better resolved in gas chromatography with no major peaks nearby to interfere with integration, cut-and-weigh procedures, or other methods of peak area measurement. In contrast, coprostanol, in thin-layer as well as gas chromatographic systems, is nearly coincident with cholesterol, a frequent contaminant of natural waters. This overlapping, combined with the excessive tailing of coprostanol, could result in inaccuracies of measuring the latter compound. The solid injection technique offers the best method for the GLC analysis of extracts containing other lipid contaminants. Not only does this method effectively eliminate interfering solvent fronts, but it also prolongs the life of the column, since solvents tend to remove some of the liquid coating off the solid support over extended periods of usage. This is particularly important for a heavily used column if we consider that for each new column, a new set of steroid standard curves has again to be established.

Although the method described in this paper is by no means simple or inexpensive, its merit is that it provides for the first time an unequivocal source of chemical data on the amounts of fecal contamination entering water. In a well-equipped laboratory, multiple analysis can be completed within a single working day once the standard curves have been prepared, thus providing an additional advantage over existing coliform methods. Most usefully, this chemical method of analysis should be combined with classical microbiological measurements, particularly for those samples where for some reason or other the coliform counting remains doubtful.

Further studies may clarify the question whether there exists any sort of correlation between bacterial and coprostanone contents in fecally polluted water samples.

ACKNOWLEDGEMENTS

This project is supported by a joint federal–provincial grant of the Ministry of Health (Project No. 604-7-677). We thank Miss S. St.-Pierre and Miss J. Thibault for their technical assistance.

REFERENCES

1 R. S. Rosenfeld, D. K. Fukushima, L. Hellman and T. F. Gallagher, *J. Biol. Chem.*, 211 (1954) 301.
2 R. S. Rosenfeld, L. Hellman and T. F. Gallagher, *J. Biol. Chem.*, 222 (1956) 321.
3 R. S. Rosenfeld and L. Hellman, *J. Biol. Chem.*, 233 (1958) 1089.
4 R. S. Rosenfeld, *Arch. Biochem. Biophys.*, 108 (1965) 384.
5 K. Hellstrom, *Acta Physiol. Scand.*, 63 (1965) 21.
6 S. Carini, *Ann. Microbiol. Enzimol.*, 14 (1964) 205.
7 E. Evrard, E. Sacquet, P. Raibaud, H. Charlier, A. Dickinson, H. Eyssen and P. P. Hoet, *Ernaehrungsforsch.*, 10 (1965) 257.
8 B. E. Gustafsson, J.-A. Gustafsson and J. Sjovall, *Acta Chem. Scand.*, 20 (1966) 1827.
9 J. J. Murtaugh and R. L. Bunch, *J. Water Pollut. Contr. Fed.*, 39 (1967) 404.
10 L. L. Smith and R. E. Gouron, *Water Res.*, 3 (1969) 141.

11 L. Tan, M. Clemence and J. Gass, *J. Chromatogr.*, 53 (1970) 209.
12 L. Tan, *J. Chromatogr.*, 45 (1969) 68.
13 *Standard Methods for the Examination of Water and Wastewater*, Amer. Public Health Ass., Washington, D.C., 13th ed., 1971, pp. 678–685.
14 R. Buttaiux, J. Samaille and Y. Pierns, *Ann. Inst. Pasteur*, 8 (1956) 137.
15 P. W. Kabler and H. F. Clark, *Amer. Water Works Ass. J.*, 52 (1960) 1577.
16 P. W. Kabler, H. F. Clark and E. E. Geldreich, *Publ. Health Rep.*, 79 (1964) 58.
17 H. F. Clark and P. W. Kabler, *Amer. Water Works Ass. J.*, 56 (1964) 931.
18 E. E. Geldreich, *Publication WP-20-3*, U.S. Dept. of the Interior, Fed. Water Pollut. Control Adm., Cincinnatti, Ohio, 1966, 122 pp.
19 W. Burrows, *Textbook of Microbiology*, Saunders, New York, 1968, Ch. 11, 20, 37.
20 H. Heukelekian and N. Dondero, *Aquatic Microbiology*, Wiley, New York, 1964.
21 A. F. Gaudy and E. T. Gaudy, *Ann. Rev. Microbiol.*, 20 (1966) 319.