7. Nicholas W. L., Hansen, E. L., Dougherty, E. C., Nematologica, 1962, v8, 129.
8. Wallis, C., Melnick, J. L., J. Bact., 1965, v90, 1632.

Received October 20, 1966. P.S.E.B.M., 1967, v124.

Improved Isolation of Anaerobic Bacteria from the Mouse Cecum By Maintaining Continuous Strict Anaerobiosis.* (31882)

RUTH W. SPEARS AND ROLF FRETER†
Department of Microbiology, Jefferson Medical College, Philadelphia, Pa.

It is well substantiated that the enteric flora contribute to the maintenance of normal physiological balance in man and animals. It is also known from numerous studies that changes in the composition of the bacterial population, and in its distribution throughout the gastrointestinal tract, are frequently associated with various pathological conditions. These subjects have been amply reviewed (1-3). However, in spite of the widespread interest in this field, little is known concerning the ecological regulating mechanisms that operate in the intestine, *i.e.*, about those factors which control whether or not, and to what extent a certain microorganism will grow in a given part of the intestinal tract(4-6). The main obstacle to studying these mechanisms is the inadequacy of most cultural methods for isolating and subculturing the strictly anaerobic Gram-negative bacteria that predominate in the large intestine(7-9).

It is well known that the commonly used technics for the culture of anaerobes, *i.e.*, those involving agar plates incubated in anaerobic jars, allow the recovery of only a small fraction of the intestinal flora(2,3,10). Nevertheless, in the absence of demonstrably better technics nearly all students of intestinal ecological regulating mechanisms chose to employ such methods. The recent studies of Bohnhoff and Miller(5) and of Schaedler *et al*(11) indicate that the most important intestinal species, *i.e.*, those which controlled the growth and distribution of Salmonellae or coliforms in the intestinal tract, were not isolated by the agar plate-anaerobic jar technics used in their work.

As part of a continuing study in this laboratory of ecological control mechanisms in the intestinal tract(6,12), attempts were made to define some of the conditions that must be met in order to achieve an improved recovery of obligate anaerobes from the normal intestinal flora of the mouse. Several variants of the agar plate-anaerobic jar technic used recently by students of intestinal ecology were therefore compared with the Hungate roll tube method(13). The results of this study are reported here.

Materials and methods. Female albino mice, approximately 28 g weight, strain CD-1, from Charles River Laboratories were used. The agar plate-anaerobic jar methods investigated were those described by Zubrzycki and Spaulding(8) and Miller and Bohnhoff(5). In preliminary studies(14) the former method proved slightly superior in terms of total bacterial counts obtained, and this was the only one used in the experiments described below. The method involved surface growth on trypticase soy agar (BBL) containing 7% sheep blood (designated TSA). The agar plates were incubated in jars containing 10%CO_2 in hydrogen in the presence of palladinized asbestos. In some experiments Eugon agar (Difco) was used as the blood agar base. Agar plates were used either freshly prepared or after storage in anaerobic jars. There was no consistent difference between fresh and anaerobically stored plates (14).

* This study was supported by contract AT (30-1)-2628 with U.S. Atomic Energy Commission, and by contract DA-49-193-MD-2307 with the U. S. Army Research and Development Command. The data are taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

† Present address: Dept. of Microbiology, Univ. of Michigan, Ann Arbor.

The roll tube medium (designated FA) was a synthetic mineral-fatty acid medium originally intended to simulate rumen fluid medium(15). It contained 0.025% Na_2S and 0.025% cysteine as reducing agents, plus 0.0001% resazurin as an oxidation-reduction indicator and was buffered with 0.4% Na_2CO_3. The final pH was 6.8. Details of preparation are described elsewhere(14). The medium was dispensed in 18 × 150 mm test tubes which were closed with rubber stoppers. As described by Hungate(13), a stream of CO_2 was directed into the tube in order to prevent entrance of atmospheric oxygen whenever the stopper had to be removed. The CO_2 used was purified by passing it over copper filings heated to 350°C. In the 2 experiments where the FA medium was used in petri dishes under 10% CO_2 in H_2 atmosphere, the concentration of carbonate buffer was reduced to 0.06% in order to maintain the proper pH of 6.8. Colony counts on *all* media were determined after 2 weeks' incubation at 37°C, except for experiments B3 and B4 (Table I) where incubation was for 4 weeks. Preliminary tests(14) had shown that there was only a negligible increase in colony counts after 2 weeks' incubation.

Three types of diluting fluids were used. (A) Trypticase soy broth (designated TSB) as used by Zubrzycki and Spaulding(8). (B) Prepared meat digest broth (designated PMB) containing 0.05% cysteine as a reducing agent and 0.0001% resazurin indicator, as used by Miller and Bohnhoff(5). The above 2 diluting fluids were dispensed in 16 × 150 mm cotton stoppered test tubes. (C) Reduced diluting fluid (designated RDF) as developed in M. P. Bryant's laboratory (1964, personal communication). This is a balanced mineral solution containing 0.05% cysteine as a reducing agent, 0.0001% resazurin indicator and 0.4% Na_2C0_3 buffer. The fluid was dispensed in 16 mm rubber stoppered tubes and was handled during manipulation under a stream of purified CO_2, in the same manner as roll tubes. Details of preparation and procedure may be found in(14). Thus, specimens diluted in RDF and cultured in roll tubes were never exposed to air at any time.

Specimens were removed immediately from mice killed by cervical dislocation. The entire cecum was clamped off at both ends and transferred to a Virtis homogenizer jar (A. H. Thomas #4285-K, 30 ml, with sealing cap assembly) containing RDF. The aerosol-tight cap of the jar was punctured by a hypodermic needle to admit a continuous stream of CO_2. Contents were removed from the cecum in the CO_2 atmosphere of the jar and homogenized at low speed (approx 1000 rpm) for 30 seconds. Serial dilutions were made immediately afterwards in several types of diluting fluids. The average weight of cecal contents thus obtained was 0.2 g. Bacterial counts reported below on a "per cecum" basis may thus be multiplied by 5 to obtain the recovery per gram of cecal material.

Total counts were performed in a Petroff-Hausser chamber (A. H. Thomas #4101-A). An appropriate dilution in RDF of the homogenized specimen was mixed with 4 volumes of phosphate buffered formalin, pH 8.1, containing 0.053% crystal violet as described in (14). This staining process resulted in good definition of the bacteria present and, possibly because of the high bacterial concentration in cecal contents, there were few structures of questionable identity.

For the differential tests shown in Table II colonies were transferred into a FA broth which was identical to the FA medium described above except that the agar had been omitted. Morphology and gram staining character were determined from growth in this broth. The basal medium for differential tests consisted of a FA broth in which the glucose, cellobiose and soluble starch had been replaced by the ingredients described below. All differential media were dispensed in rubber stoppered tubes which were gassed with purified CO_2 whenever opened, except for sugar fermentation tests in which 10% CO_2 in N_2 was used:

(a) Gelatin liquefaction. Basal medium was used containing 5% gelatin (Difco), 0.1% glucose and 1% trypticase. Cultures were incubated at 37°C for a maximum of 15 days. Liquefaction was determined after storage at 4°C for 1 hour.

(b) Sugar fermentations. Basal medium contained 0.5% glucose or lactose. In order

to reduce the buffering capacity of the medium, the content of Na_2CO_3 in the basal medium was reduced to 0.06% and the gas used was 10% CO_2 in N_2 (rather than the usual 100% CO_2). The sterile medium had a pH of 6.8. Final pH was determined after 8 days growth at 37°C. A drop of 0.2 pH units or more may be regarded as indicating significant acid production.

(c) Motility and H_2S production. The basal medium contained 0.5% agar (Difco), 0.1% glucose, 0.05% ferric ammonium citrate and 0.008% sodium thiosulfate. Final reading was made after 6 days incubation at 37°C. Motility was confirmed by dark-field observation of hanging drops from 18-24 hour cultures in FA broth.

Results. Table I shows the results of 8 experiments comparing the effect of 3 diluting fluids and of 4 culture methods on the total number of bacteria that could be recovered from the mouse cecum. In each experiment 2 parallel series of dilutions were prepared in different fluids. Each dilution of these series was used to inoculate 10 replicate roll tubes containing FA medium plus 5 replicate agar plates as indicated in the table. As may be seen in Table I, optimal recovery of bacteria was obtained only when the entire process of handling, diluting and culturing of the specimen was carried out in a reduced medium and under exclusion of oxygen (RDF diluent and roll tubes). The recovery under these conditions ranged between 19.7 and 33.7 of the total (Petroff-Hausser) count. If a diluting fluid such as PMD was used which did contain a reducing agent but which was dispensed in cotton stoppered tubes (and therefore exposed to oxygen), the recovery dropped to 13.7-21.3% (PMD and roll tubes). The least efficient recovery was obtained when simple trypticase soy broth in cotton stoppered tubes was used as the diluting fluid (2.14-3.8% with TSB and roll tubes). As shown in Table I, these differences were statistically highly significant.

In contrast to roll tubes, anaerobic plates always gave a low recovery of bacteria even when the best diluent, RDF, was used (0.5-8.2% of the total count). The effect of the various diluents on recovery of bacteria on plates was similar but less pronounced as compared to that observed with roll tubes, *i.e.,* RDF gave optimal recovery and TSB the least. When fatty acid medium (the same as used in roll tubes) was dispensed in the form of agar plates, the recovery was even lower than that obtained on blood agar plates. This was true when the gas phase in the anaerobic jars contained the usual 10% CO_2 in H_2 or when the jars were filled with 100% CO_2 (the same atmosphere as used in roll tubes). Consequently, the inferior recovery on agar plates was not due to the medium or the gas phase employed, but must be attributed to the fact that the bacteria streaked on plates were exposed to oxygen until the plates were stored in anaerobic jars. In different experiments the time between plating the diluted specimen and complete removal of air from the anaerobic jar varied between 20 and 45 minutes.

The results shown in Table I raised the question whether or not the higher counts obtained with the RDF-roll tube combination indicated a qualitative as well as a quantitative difference, *i.e.,* whether some bacteria isolated by this procedure did not grow at all on anaerobic plates. The following experiment was designed to test this.

The cecal contents of one mouse were diluted in RDF and cultured in (a) 10 replicate roll tubes containing FA medium and (b) 5 replicate blood agar plates (TSA). The recovery was as follows: Total (Petroff-Hauser) count—1.8×10^{11} per cecum; roll tubes—6.1×10^{10} (= 34% of total); blood agar plates—2.5×10^{9} (= 1.39% of total). Subcultures were made from both media into liquid fatty acid medium and on blood agar plates. All 26 colonies picked from the roll tubes and 24 of 25 picked from the plates could be subcultured. Of these, 17 proved to be capable of growth on aerobic blood agar plates. The remaining anaerobic strains were carried through a series of differential tests, the results of which are shown in Table II. As may be seen, there were 26 groups of bacteria among the 33 anaerobes isolated. Most noteworthy seems the fact that 10 of the strains isolated from roll tubes did not grow

TABLE I. Effect of Diluent and Culture Method on Total Viable Counts of Bacteria Recovered from the Mouse Cecum.

		Roll tubes (FA-medium)		Anaerobic plates				Significance roll tubes *vs* plates (with RDF)§	Significance of diluent§	
Exp No.	Diluent*	Avg viable count†	% of PH count‡	Medium*	Gas used	Avg viable count†	% of PH count‡		In roll tubes	On plates
A1	RDF	130	19.7	TSA	10% CO_2	49.2	7.4	<.001	<.001	NS
	TSB	24.6	3.8			37.8	5.8			
A2	RDF	70.6	20.3	EuA	10% CO_2	11.8	3.4	<.001	<.001	<.05
	TSB	7.5	2.14			6.8	2.0			
A3	RDF	196	23.2	EuA	10% CO_2	18.8	2.2	<.001	<.005	<.005
	PMD	118	13.7			9.4	1.1			
A4	RDF	101	26.3	TSA	10% CO_2	31.0	8.2	<.001	<.001	<.005
	PMD	69.2	18.2			18.6	5.0			
B1	RDF	76.0	21.7	FA	10% CO_2	10.4	2.8	<.001	<.001	NS
	TSB	13.0	3.7			4.8	1.4			
B2	RDF	292	33.7	FA	10% CO_2	14.2	1.63	<.001	<.01	NS
	PMD	111	13.9			13.2	1.51			
B3	RDF	179	27.2	FA	100% CO_2	8.8	1.3	<.001	<.001	NS
	TSB	18.0	2.7			9.4	1.4			
B4	RDF	98.0	25.8	FA	100% CO_2	2.0	0.5	<.001	NS	NS
	PMD	81.0	21.3			1.4	0.4			

* RDF = reduced diluting fluid; TSB = trypticase soy broth; PMD = prepared meat digest broth; TSA = trypticase soy blood agar; EuA = Eugon blood agar; FA = fatty acid medium.

† Counts shown × 10^{-8}, per content of one cecum, based on 8-10 replicate roll tubes or 5 replicate plates.

‡ Based on total number of bacteria in the specimen determined by Petroff-Hausser count.

§ Analysis of variance(16) after logarithmic transformation of each observation.

TABLE II. Comparison of Anaerobes Isolated in Roll Tubes and on Anaerobic Plates.

Strain designation*	Plate growth†	Gram stain	Morphology‡	Motility	Gelatin liquefaction	Producer of Gas	Producer of H_2S	Final pH in§ Glucose	Final pH in§ Lactose
3T, 12T, 13T, 19P	+	—	3	—	—	—	+	6.7	6.6
3T, 4T	—	—	6	—	—	—	—	6.6	6.6
26T, 16P	+	+	1	—	+	+	+	6.4	6.7
15T, 9P	+	—	2	—	+	—	+	6.3	6.3
17T	—	—	6	—	—	+	+	6.6	6.5
14T	+	+	2	—	+	—	+	6.1	6.0
25T	+	+	4	—	+	+	+	6.2	6.2
1T	—	—	1	—	—	—	+	6.7	6.7
2T	—	—	2	—	—	—	—	6.6	6.5
16T	—	—	3	—	—	—	+	6.3	6.6
23T	—	—	3	+	—	+	—	6.6	6.7
11T	—	—	4	—	—	—	—	6.2	6.3
19T	—	—	5	+	—	+	—	6.6	6.6
5T	—	—	6	—	—	+	—	6.6	6.7
17P, 20P	+	—	3	—	+	—	+	6.0	5.8
11P	+	+	1	—	+	+	+	6.2	6.3
18P	+	—	5	—	—	—	—	6.3	6.4
1P	+	+	2	—	—	—	—	6.6	6.6
3P	+	—	1	—	—	+	+	5.8	5.7
13P	+	—	1	—	+	+	+	5.9	5.7
5P	+	—	5	—	—	+	+	6.6	6.4
14P	+	—	2	—	+	—	—	6.0	6.0
2P	+	—	3	+	—	—	—	6.0	6.2
7P	+	—	3	—	—	—	—	6.7	6.6
15P	+	—	3	—	—	—	—	4.9	6.6
12P	+	—	4	—	+	+	+	5.9	5.7

* T = originally isolated in roll tube; P = originally isolated on anaerobic plate.
† + indicates that subcultures grew on anaerobic blood agar.
‡ 1 = cocci or coccobacilli; 2 = short rods; 3 = bipolar or granular (short to medium) rods; 4 = medium to long rods; 5 = slender rods, some pleomorphic; 6 = slender rods in long or short chains, also singly.
§ pH of sterile medium was 6.8.

on blood agar plates (Table II), even after repeated attempts at subculture on this medium. In contrast, all strains regardless of source could be subcultured in roll tubes on fatty acid medium.

Discussion. As reported elsewhere(14) the precise conditions used in the present work for the roll-tube and anaerobic jar methods were optimal as determined in extensive preliminary studies. Recovery of bacteria on anaerobic blood agar plates (using TSB or PMD diluent) was in the range of 3.5×10^9 to 1.9×10^{10} per gram of wet cecal content (based on an average weight of 0.2 g per cecal content). This compares well with results reported by others for mouse large intestine: Gibbons *et al,* 2.1-16.9 $\times$ 10^9 per gram(17); Smith and Crabb, 3×10^9 per gram(9); Schaedler *et al,* approximately 10^9 per gram(11). Zubrzycki and Spaulding(8) report values averaging 1×10^9 per gram of human feces. Our total recovery of organisms by the roll tube method (19.7-33.7% of total count) compares to that reported by Bryant(18) for rumen bacteria (10-30%).

The data presented indicate that a method which provides anaerobiosis only during incubation of cultures is likely to be insufficient for the strict anaerobes, *i.e.,* for those bacteria which in the present study constituted the most numerous population in the large intestine of the mouse. Improved results were obtained only when the specimen was never exposed to oxygen at any stage of the procedure (RDF diluent with roll tubes). Under these conditions, several bacterial strains were isolated which could not be subcultured at all by the plate-anaerobic jar method. Many species of the mouse cecal flora thus exhibited a degree of sensitivity to transient contact with oxygen which is similar to that of microorganisms from the bovine rumen. This may explain the findings of earlier investigators (5,11) that mixtures of bacteria isolated from normal mice were unable to re-establish the functions of a normal enteric flora in germ-

free or antibiotic treated animals while, at least in one instance(11), suspensions of fecal pellets could do so.

As discussed above, there were 10 strains isolated in roll tubes which did not grow at all on agar plates. In turn, most organisms isolated by the plate-jar method were different from those isolated in roll tubes (Table II) even though they could be subcultured by the roll tube method. Most likely, these are moderately oxygen-sensitive microorganisms which in the roll tubes were overgrown by the more numerous strict anaerobes. While other explanations cannot be ruled out entirely, these results are consistent with the assumption that the plate- anaerobic jar method was selective for a relatively small population of bacteria having somewhat less fastidious requirements for anaerobiosis than the predominant anaerobes.

In view of the above it seems likely that drastic changes from previously used technics will be necessary before it becomes possible to conduct more meaningful studies of microbial ecology in the mouse intestine, and probably also that of man and other animals. While the roll tube method is rather cumbersome for large scale or routine studies, anaerobic chambers, based on the model described by Rosebury(19), may perhaps provide a simpler means for obtaining the complete exclusion of oxygen that was the principal requirement for improved recovery in the present work. Relevant studies are presently in progress. It may also be pointed out that the FA medium for roll tubes was employed in the present study simply because it had been found by others to give good results with rumen bacteria. It is entirely possible that other media, dispensed in roll tubes, may improve the recovery rates reported here.

Summary. Two technics for anaerobic culture were compared for their efficiency in isolating anaerobic bacteria from the cecum of the mouse. A conventional method using a broth diluent and blood agar plates incubated in anaerobic jars recovered only 1.1-5.8% of the total number of bacteria present. In contrast, 19.7-33.7% of the total bacterial count was recovered when the specimen was processed by the Hungate technic, which entailed anaerobic collection, dilution in reduced fluid under CO_2 and culture in roll tubes. Many of the bacteria isolated in this manner did not grow at all on blood agar plates incubated in anaerobic jars. The data presented indicate that the predominant, strictly anaerobic bacteria of the cecal flora were sensitive to even transient contact with atmospheric oxygen.

The authors are most grateful to Dr. M. P. Bryant and his staff for a detailed demonstration of the Hungate roll tube technic.

1. Luckey, T. D., Adv. Appl. Microbiol., 1965, v7, 169; Symposium, Am. J. Dig. Dis., 1965, v10, No. 10.
2. Donaldson, R. M., New Engl. J. Med., 1964, v270, 938, 994, 1050.
3. Dubos, R., Schaedler, R. W., Fed. Proc., 1963, v22, 1963.
4. Dixon, J. M. S., J. Path. Bact., 1960, v79, 131; Meynell, G. G., Brit. J. Exp. Path., 1963, v44, 209; Branche, G. H., Young, V. M., Robinet, H. G., Massey, E. D., Proc. Soc. Exp. Biol. and Med., 1963, v114, 198.
5. Bohnhoff, M., Miller, C. P., Martin, W. R., J. Exp. Med., 1964, v120, 805, 817; Miller, C. P., Bohnhoff, M., J. Infect. Dis., 1962, v111, 117.
6. Freter, R., J. Infect. Dis., 1962, v110, 38.
7. Eggerth, A. H., Gagnon, B. H., J. Bact., 1933, v25, 389; Smith, H. W., J. Path. Bact. 1965, v89, 95.
8. Zubrzycki, L., Spaulding, E. H., J. Bact., 1962, v83, 968.
9. Smith, H. W., Crabb, W. E., J. Path. Bact., v82, 53.
10. Dehnert, J., Wien. Klin. Wschr., 1961, v73, 43, 729.
11. Dubos, R., Schaedler, R. W., Costello, R., Hoet, P., J. Exp. Med., 1965, v122, 67; Schaedler, R. W., Dubos, R., Costello, R., J. Exp. Med., 1965, v122, 77.
12. Ozaawa, A., Freter, R., J. Infect. Dis., 1964, v114, 235.
13. Hungate, R. E., Bact. Rev., 1950, v14, 1.
14. Spears, R. W., Doctoral dissertation, 1966, Jefferson Medical College, Philadelphia.
15. Bryant, M. P., Robinson, I. M., J. Dairy Sci., 1963, v46, 150.
16. Winer, B. J., 1962, Statistical Principles in Experimental Design, McGraw-Hill, New York.
17. Gibbons, R. J., Socransky, S. S., Kapsimalis, B., J. Bact., 1964, v88, 1316.
18. Bryant, M. P., U.S.D.A. ARS, 1961, 44.

19. Rosebury, T., Reynolds, J. B., Proc. Soc. Exp. Biol. and Med., 1964, v117, 813.

Received December 5, 1966. P.S.E.B.M., 1967, v124.

Renal Acid Deoxyribonuclease: Observations on Distribution and Cell Relationship. (31883)

SEYMOUR ROSEN, MARY COUGHLAN AND KEVIN G. BARRY
(Introduced by William S. Gochenour, Jr.)
Department of Metabolism, Division of Medicine, Walter Reed Army Institute of Research, Washington, D. C.

Acid deoxyribonuclease is a recognized member of the lysosomal enzyme group(1). It has been extensively investigated in the liver(1,2,3), but has received less study in kidney tissue(4,5). Straus(6,7) utilizing kidney cortical homogenates, isolated 3 fractions sedimenting between 650 and 15,000 $\times$ *g*, rich in acid deoxyribonuclease, and similar in enzyme content to deDuve's liver cell lysosomes(1). Interestingly, the acid deoxyribonuclease activity in these fractions responded differently to activation by osmotic effects. This report presents further evidence in this regard and illustrates the striking regional distribution of acid deoxyribonuclease in the rat kidney.

Materials and methods. Male Wistar rats weighing between 350 and 500 g were sacrificed by decapitation and exsanguination. The kidneys were quickly removed, washed in saline, and their capsules stripped. A 5.5 mm cork borer was inserted midway between the renal poles in such a manner as to completely include papilla. The core thus obtained was divided into 4 segments: outer cortex (O.C.), inner cortex (I.C.), outer medulla (O.M.), and inner medulla (papilla, I.M.). In a previous study(8), this method for separating the renal segments is described in detail. In each experiment, segments from 10 kidneys were combined. The tissue was homogenized in 0.25 M sucrose (1:20 w/v) using 5 uniform passes with a teflon pestle (Potter-Elvehjem Homogenizer) at 2000 RPM. A portion was centrifuged for 30 minutes at 37,000 $\times$ *g* in a refrigerated centrifuge (Servall Superspeed RC-2 with an S-34 angle head). The remainder of the homogenate was frozen and thawed 10 times for optimal enzyme activation(9). Such a procedure did not alter the activity of the supernatant.

For the distribution experiment, 300 μliters of homogenate (after freeze-thaw activation) or supernatant were added to an equal volume of incubation media containing 1 mg DNA/ml (calf thymus, highly polymerized, Mann Research Laboratory, New York), 0.2 M acetate buffer, pH 5.0, and 20 mM $MgCl_2$(2,10). The samples were incubated for 10 minutes at 37°C. The reaction was stopped by addition of 600 μliters of 10% $HClO_4$. The tubes were then centrifuged, the supernatant removed, and absorption at 260 mμ was determined. Appropriate controls were done. The effects of molarity were examined by decreasing the homogenate volume to 200 μliters and adding 100 μl of either water or 1.0 M sucrose, resulting in a final sucrose molarity in the digestion mixture of 0.08 M or 0.25 M, respectively. At the same time, the effect of freeze-thaw techniques was also investigated. Supernatant and homogenate protein was determined by the method of Lowry(11). Enzyme activity was calculated both as specific (millimicromoles/minute/mg protein) and total (millimicromoles/minute/ml of 5% homogenate) activity. The units were in terms of liberated mononucleotides (2,12) assuming an average extinction coefficient of 8.5 $\times$ 10^6 cm^2 $mole^{-1}$. The results were calculated as the mean and standard deviation of 10 experiments; when appropriate, the Student's t-test (paired) was employed.

Results and discussion. When calculated as total activity (Fig. 1) the O.C. had the