that afferent pathways from the diencephalic reticular ascending system induce, via hypothalamic centers, neurohumoral mechanisms participating in stress situations, including increased liberation of adrenalin. The lesion of the thalamic mid-line nuclei apparently interrupts this neurohumoral reflex arc which participates in metabolic preparatory reactions.

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Synchronization of Division in Escherichia coli

Abstract. The smallest cells of a culture in the logarithmic phase, when isolated by a single rapid filtration through 1.2-μ Millipore paper, show good synchronization and reproducibility through the first division cycle. The method minimizes metabolic shock and provides a culture in which an easily reproducible initial state is established at a known time.

Tests of the Maruyama and Yanagita technique (*1*) for isolating the largest cells of an *Escherichia coli* culture led us to the conclusion that temperature shock, introduced by extensive manipulations at room temperature, was probably responsible for the erratic results obtained. Subsequent experiments led to a synchronizing technique which is simple and fast and involves no operations likely to disturb the normal metabolism of the organism.

Our procedure for *E. coli* strains K12 (λ) and B is as follows (*2*). A 100- to 150-ml batch culture is grown in synthetic medium (*3*), with continuous aeration, to a density of the order of 10^8 cells per milliliter. Without other manipulation, the culture is filtered quickly through a single sheet of grade RA Millipore paper, pore size 1.2 μ, standard Millipore equipment being used for vacuum filtration (*4*), with a 25-liter bottle as "vacuum reservoir." Filtration is interrupted when a few milliliters of culture remain on the filter or when clogging reduces the rate of filtration appreciably. Filtration is completed in 2 to 3 minutes. The filtrate, containing 1 to 2 percent of the total cell population, is aerated through a sintered glass wand immediately after filtration. The entire process is carried out in the constant-temperature cabinet in which the initial culture is grown. No significant improvement resulted from repeated filtration through single sheets of Millipore paper or from the use of stacks of two or more papers.

Figure 1 shows the results of a typical experiment in which the filtrate was sampled periodically and assayed for cell count by triplicate plating on nutrient agar. Dilutions were adjusted to yield about 100 colonies per plate at minimal density. Zero time is taken at the mid-point of the filtration interval. The results of all our tests, with the two strains of *E. coli* and temperatures ranging from 25° to 37°C, may be summarized as follows. (i) Through the first growth cycle the $n(t)$ curve (cell count–time curve) is flat for about 70 percent of the mean generation time. The doubling time approximates the mean generation time closely. Contrary to common experience with temperature shock methods, the doubling is real rather than nominal, supporting the conclusion that quick filtration without separation of the cells from the original medium involves little if any disturbance of normal growth. (ii) Under close temperature control the second division cycle is well marked, but synchronization deteriorates in this cycle. Beyond 1.5 times the mean generation time, reproducibility of the $n(t)$ curve has not been good enough to warrant selection of a typical curve. Relaxation of temperature control is reflected in a deterioration of synchrony and reproducibility in the interval from 0.7 to 1.5 times the mean generation time. (iii) Beyond the second cycle the $n(t)$ curve assumes a form which approximates the normal growth curve.

As additional grades of Millipore paper become available, improvement in cell-size resolution, with consequent sharpening of synchronization, will probably be possible. It should be remarked, however, that if the standard deviation in generation time characteristic of *E. coli* is as large as 0.3, as was recently estimated (*5*), a cell population strictly homogeneous in age at filtration would show rapid deterioration of synchrony after the first cycle. Attempts made in our laboratory by W. V. Morgan and one of us

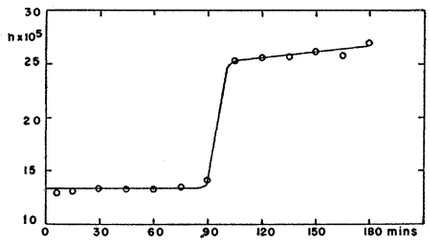

Fig. 1. Cell count-time characteristic for *E. coli* K12 (λ) filtered at zero time. *N*, cells per milliliter; *t*, 30°C; mean generation time, 105 minutes. Grade RA Millipore paper was used as filter.

(P.A.A.) to impose synchronized division on continuously cultured *E. coli* by programmed temperature cycling gave disappointing results which are probably attributable to this factor.

From the preparative standpoint, the quick-filtration method would appear to have the marked advantage of supplying a culture for which the initial state and succeeding growth curve are sharply defined on the time axis and hence determinable before rather than after the event (*6*).

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Utilization of Organic Carbon by a Marine Crustacean: Analysis with Carbon-14

Abstract. An isotope-dilution technique using carbon-14 was employed to determine quantitatively the carbon budget of a filter-feeding crustacean. The amount of carbon ingested ranged between 0.044 and 0.139 mg. Incorporation of carbon varied between 11.3 and 73.6 percent per day per organism, with an average of 32.5 percent for the animals tested. Values for oxygen consumption are given as they relate to carbon intake and utilization.

Energy and feeding interrelationships have been experimentally determined for some zooplankters. For example, Richman (*1*) determined a calorimetric budget for the fresh water crustacean

Daphnia pulex, and Marshall and Orr (*2*) used radioactive carbon to determine the filtering rates and the percent of food digested by the marine copepod, *Calanus finmarchicus*. The latter authors made no attempt at a quantitative measure of carbon in their experiments. This report presents a method which utilizes an isotope-dilution technique for quantitative measurements of the utilization and egestion of particulate organic carbon by a planktonic filter-feeding marine crustacean, *Euphausia pacifica*.

The experimental animals (*E. pacifica*) were collected in net hauls (at a depth of about 250 m) off the coast of La Jolla and were brought back at 10°C to the laboratory within a few hours.

A period of 24 hours was usually allowed for the unfed captive animals to void particulate matter from the gut. Eight-day-old, 50-ml, bacteria-free cultures of a green algal flagellate, *Dunaliella primolecta*, grown in a sea water medium (*3*), were labeled with C^{14} (10 μc of $NaHC^{14}O_3$) for 3 to 4 days and were then supplied as food. The amount of carbon in the food offered to the animals in the laboratory ranged between 0.117 and 0.313 mg.

On the day of an experiment the algae were collected by centrifugation, washed free of soluble C^{14}, and suspended in sea water previously filtered through Millipore membranes (0.45 μ porosity). Total organic carbon and C^{14} measurements were determined for aliquots of the algal culture. This information provided the proportion of total carbon to C^{14} in the algae. The culture was suitably diluted to give approximately 0.1 to 0.3 mg of algal carbon in a vessel (about 1 to 3 $\times$ 10^5 cells per milliliter). Each vessel was then supplied with an adult euphausiid (dry weight 3 to 5 mg). When euphausiids are put into a heavy suspension of algae, that is, enough cells to tint the sea water green, they quickly filter out enough algae to completely fill the intestine, digestive gland, and crop. Control bottles without animals were included to ascertain the amount of algal respiration. Bottles were usually of 65-ml capacity and contained 40 ml of fluid, but occasionally larger bottles were used. At the start of the experiment an aliquot of the sea water containing the radioactive algae was removed to determine the quantity of organic carbon available to the animals. The method of Kay (*4*) was used for measurement of organic carbon. Another aliquot of algae was retained on a Millipore filter (pore size, 0.45 μ) and analyzed for C^{14}.

The experimental bottles and the contents were kept in the dark at 10°C for 24 hours. Then the animals were removed, washed in filtered sea water, and dissected to remove both their intestinal tracts and digestive glands. Fecal pellets were removed from the water with a pipette and washed gently. Finally, another aliquot of the algae was taken out and filtered for C^{14} determination.

Table 1. Fractionation of carbon by radioactivity. In this experiment, the carbon for each fraction was calculated by use of the ratio

$$\frac{27.9 \times 10^3 \text{ cpm}}{0.174 \text{ mg C}} = \frac{\text{Observed cpm}}{x}$$

where cpm is counts per minute.

Material analyzed	10^3 cpm	Total C (mg)
Time: 0 hours		
40 ml Dunaliella culture	27.9	0.174
Time: 24 hours		
40 ml Dunaliella culture	5.07	0.032
Ingested (by difference)		0.142
Egested in fecal pellets	1.27	0.008
Eviscerated euphausiid	16.7	0.104
Catabolized (by difference)		0.030

After drying, each eviscerated euphausiid was weighed, combusted to carbon dioxide, and assayed for total carbon (*4*). The mean of 16 carbon determinations is 34.6 percent on a dry weight basis (range 25.4 to 39.0 percent). The CO_2 thus produced was recovered as $BaCO_3$ and counted for C^{14} radiation in a windowless proportional counter (Nuclear Measurements Corporation). The amount of C^{14} activity in the barium carbonate was corrected against a self-absorption curve. The radiation emitted by the radioactive algae was also counted. No self-absorption correction was made for filtered algae because of the extremely low weight of the sample and the lack of measurable absorption of the C^{14} radiation (*5*).

Knowing the C^{14}/total C ratio of an algal sample permits the calculation of the total carbon contributed by the algae, as shown in Table 1. The constancy of the ratio C^{14}/total C was tested. It appears that there usually is a change, due to respiratory processes, of -4 to -5 percent in this ratio over the experimental period. Table 1 presents the results of a typical experiment.

Table 2 gives the carbon balances calculated from the C^{14}/total C ratios found for eight animals at different phytoplankton densities. Also shown are the calculated efficiencies with which these animals were able to accumulate carbon in their tissues through digestive processes.

Respired CO_2 was not determined directly but was estimated by difference. An indirect gasometric method was used also to calculate the magnitude of total respiratory carbon for comparison with the C^{14} method. Oxygen consumption was found to be linear over several hours at 10°C by tests with the water analyzer devised by Scholander *et al.* (*6*). Q_{O_2} values were based upon eight analyses of individual animals, and showed a range of 0.85 to 1.52 ml/gm per hour, from which the mean of 1.14 was derived. On the assumption that the respiratory quotient was 1.0, CO_2 evolution was calculated from the average oxygen consumption, and was found to have a reasonably close correspondence in some cases with data collected by the C^{14} method (animals Nos. 1 to 4, Table 2).

Some animals (Table 2) showed a larger catabolic carbon fraction than could be accounted for by respiratory processes alone. It is probable that catabolic carbon compounds other than CO_2 are excreted by the animals.

From Table 2 it is also seen that large variations may exist in the efficiency of these zooplankters to incorporate ingested carbon. In the animals tested, this ranged between 11.3 and 73.6 percent. However, the fate of the incorporated carbon was not ascertained. These data may reflect depo-

Table 2. Carbon budget of *Euphausia pacifica*. All weights are given in milligrams.

No.	Dry wt. of animal	Wt. C in eviscerated animal	% C in animal	Wt. algal C offered to animal	Wt. C ingested by animal	Wt. C incorporated	% C incorporated	Wt. C of gut and gland	Wt. C egested	Wt. catabolic C, including CO_2	Wt. C respired (calculated from RQ = 1)
1	4.6	1.66	36.6	0.174	0.139	0.102	73.6	< 0.001*	0.009	0.029	0.033
2	3.7	1.22	33.0	0.156	0.057	0.018	31.6	0.005	0.006	0.028	0.026
3	3.0	1.01	33.6	0.156	0.056	0.021	37.8	0.003	0.007	0.025	0.021
4	3.9	1.49	38.2	0.117	0.044	0.005	11.3	< 0.001*	< 0.001*	0.039	0.028
5	4.1	1.60	39.0	0.276	0.097	0.015	15.5	0.003	0.001	0.079	0.029
6	4.6	1.74	37.8	0.313	0.113	0.020	17.8	0.002	0.002	0.065	0.033
7	3.4	1.27	37.4	0.313	0.147	0.062	42.2	0.011	0.002	0.073	0.024
8	5.1	1.62	31.8	0.313	0.101	0.029	29.7	0.006	0.001	0.065	0.035

* Weight not determinable by the method used.

sition of storage products or actual growth.

The effect of varying temperatures on oxygen utilization was tested gasometrically. The euphausiids were found to have a Q_{10} of approximately 2 between 5° and 12°C. This would imply that when these animals cross the thermocline into warmer water their basal carbon needs increase. However, except those by Fox *et al.* (*7*), few published data exist on the amount of particulate carbon available for zooplankters in the sea.

Application of the method presented in this paper to other organisms, with proper attention to the experimental variables cited here, may permit a direct measurement of the carbon flow through biological systems in aquatic environments. Future investigations call for studies of relative nutritional states and comparative proportions of stored reserves in experimental animals (*8*).

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Toxicity of Bacterial Exotoxins by the Oral Route

Abstract. Reports to the contrary notwithstanding, both tetanus and diphtheria toxins are demonstrably orally toxic. The significance of this finding is considered in relation to the definition of those factors which are determinants of the potency of bacterial protein toxins by the oral route.

The statement has often been made that among the classical bacterial protein exotoxins, that is, diphtheria, tetanus, and botulinum, only the latter is poisonous by the oral route (*1*). Thus, it is tempting to attribute the orally toxic nature of botulinal toxin to characteristics unique to this toxin. Recently a different point of view has been evolving (*2*). Toxicity of proteins by the oral route can be conceived as resting on the fact that the intestinal barriers to systemic absorption are imperfect and permit the escape of small amounts of protein. If this hypothesis is correct, one might expect to find that in addition to botulinal toxin, other proteins which are poisonous in extremely small amounts are potentially oral poisons.

A logical candidate for a test of the concept is tetanus toxin, a simple protein, whose potency by parenteral routes is of the same order of magnitude as the type A botulinal toxin. We have, therefore, tested for the oral toxicity of tetanus toxin in spite of the negative reports in the bacteriological literature.

Employing the Namru strain of white mouse, three separately prepared batches of crude tetanus toxin, kindly supplied by a commercial source from production runs, proved to be orally toxic. A typical experience is recorded in Table 1. The specificity of deaths was confirmed by observation of the protective value of specific antitoxin. Repeated titrations showed that between 200,000 and 1,200,000 times the intraperitoneal LD_{50} was required for one oral LD_{50}. With the same strain of mice and crystalline type A botulinal toxin, 50,000 to 250,000 intraperitoneal LD_{50} are needed for an oral LD_{50} (*3*).

The tetanus toxin also proved capable of killing large guinea pigs (800 gm) when 600,000 or more intraperitoneal lethal doses were given an individual animal *per os*.

Tests were also performed with crude diphtheria toxin. An occasional Namru strain mouse was poisoned orally. The oral toxicity for guinea pigs was irregular in occurrence and required the use of volumes (>3 ml) of the toxic fluid near the practical limits for *per os* injections. Consequently, it did not prove practical to collect data for calculation of oral LD_{50} values. The sporadic nature of the observations of oral toxicity for the mouse by diphtheria toxin perhaps is not surprising, since mice are thousands of times less sensitive to the toxin parenterally than are guinea pigs (*4*). In the future it will be desirable to test more highly concentrated preparations of the diphtheria toxin than have been available for the present study: 6000 (crude toxin lot 65316 from Merck, Sharp and Dohme) and 30,000 (ultrafiltrate lot Rx 057435-437 Parke, Davis and Co.) intraperitoneal LD_{50} for the guinea pig per milliliter.

Can the reported lack of oral toxicity for tetanus and diphtheria toxins be reconciled with our positive findings? The answer may relate to the fact that unlike the situation for *Clostridium botulinum,* laboratory cultures of tetanus and diphtheria bacilli generally do not produce concentrations of toxin in a volume of fluid that is feasible to inject into the alimentary tract of small laboratory animals as a fatal single dose. In our experience with such material, a number of oral doses separated in time must be given in order to be able to introduce without regurgitation and loss of the injected preparation enough toxin for fatalities to occur. I believe that negative reports result from trials with single injections of insufficiently concentrated toxin. When concentrated toxin preparations are tried, I predict a much larger number of bacterial toxins than are presently known will prove to be orally toxic. In this event, the reputation of botulinal toxin as an oral poison must rest not on any single intrinsic property of the toxin molecule, but rather on the particular ecological circumstances which permit the presence, growth, and toxin production of *C. botulinum* in a variety

Table 1. Toxicity of tetanus toxin in mice of 18 to 20 gm weight by intraperitoneal and *per os* administration. The preparation employed was a crude culture filtrate kindly made available by a commercial source from a routine production run in which the Harvard strain of tetanus bacillus, grown in a protein digest medium, was used. The filtrate was potent enough to permit the oral introduction of a fatal dose in a volume of fluid well tolerated by the mouse. The technique of *per os* injection has been reported and does not result in macroscopic evidence of tissue trauma (*3*). With this preparation of toxin, 3 ml (but not less), when given orally to guinea pigs of 800 to 1000 gm weight, resulted in death. Eight control mice received 500 units of antitoxin and were then injected with 0.5 ml of undiluted toxin *per os*; none of these died.

Intraperitoneal		*per os*		
Dose and Dilution	Deaths/No. injected	Dose and Dilution	Deaths/No. injected	
			Mice not starved	Mice starved 18 hr
0.5 ml, 1×10^5	4/4	0.5 ml, undiluted	9/10	9/10
0.5 ml, 2×10^5	4/4	0.25 ml, undiluted	7/10	6/10
0.5 ml, 4×10^5	4/4	0.25 ml, 2-fold	2/10	2/10
0.5 ml, 8×10^5	0/4	0.25 ml, 4-fold	3/10	1/10
0.5 ml, 16×10^5	0/4	0.25 ml, 8-fold	1/10	1/10