References and Notes

1. C. P. Swanson, *Science* **107**, 458 (1948).
2. H. J. Muller, *Radiation Biol.* **1**, 475 (1954).
3. J. MacKey, *Hereditas* **40**, 65 (1954).
4. L. J. Stradler, *Proc. Natl. Acad. Sci. U.S.* **15**, 876 (1929); K. Fröier, *Hereditas* **32**, 297 (1946); A. Gustafsson, *ibid.* **33**, 1 (1947).
5. E. R. Sears, *Missouri Univ. Agr. Expt. Sta. Bull. No. 572* (1954).
6. ———, *Wheat Inform. Serv.* **6**, 1 (1957).

7. We are indebted to B. P. Pal for advice and encouragement in this study.

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Carbon Dioxide Fixation in Marine Invertebrates: A Survey of Major Phyla

Abstract. Fourteen species of marine invertebrates representing 12 phyla were kept in sea water containing $NaHC^{14}O_3$ for 1 hour. All of them fixed CO_2 into acids of the Krebs citric acid cycle. In most species the major portion of the radioactivity recovered after chromatography was in succinic, fumaric, and malic acids. The findings favor the hypothesis that both CO_2 fixation and the citric acid cycle are virtually universal among marine invertebrates.

The finding of the reaction of CO_2 with propionate to form succinate in isolated tissue of the oyster (*1*) is one of the few demonstrations of CO_2 fixation in invertebrates. The only other marine invertebrate which has been investigated is the developing sea-urchin egg, which incorporates $C^{14}O_2$ into several fractions of organic material (*2*). In order to determine the extent of CO_2 fixation among marine invertebrates, animals representing 12 phyla were examined in this study (*3*).

The animals used in the experiments reported here were collected in the vicinity of Beaufort, North Carolina, during the summer months, and were used immediately after collection (*4*). After cleaning and weighing, whole animals or pieces of tissue were incubated in stoppered 125-ml flasks with filtered sea water to cover them. From 5 to 120 μc of $NaHC^{14}O_3$ were added from a syringe, giving concentrations in the medium of 0.5 to 5.3 μc/ml in all except the sponge. Details of each experiment are recorded in Table 1. Metabolic activity was halted after 60 minutes of incubation by placing the living material in cold acetone, and homogenization was begun at once. Organic acids were extracted according to the method of Frohman, Orten, and Smith (*5*), chromatographed with known acids on paper, and counted as described previously (*1*). The relative map positions of the acids agreed quite well with those reported by Carles *et al.* (*6*) for similar solvent systems.

The results of 14 experiments are

Table 1. Conditions of experiments on uptake of $NaHC^{14}O_3$ by marine invertebrates. Incubation time was 60 minutes in each case.

Species	No. of specimens	Fresh wt. (gm)	Volume of sea water (ml)	$NaHC^{14}O_3$ (μc)	Temp. (°C)
Hymeniacidon heliophila (sun sponge, Porifera)	Several branches	1.50	8.5	120	23.5
Aiptasia pallida (anemone, Cnidaria)	40	9.0	5.2	20	28.0
Bdelloura candida	150	0.53	6.0	32	28.0
Stylochus zebra (flatworms, Platyhelminthes)	1	0.07	1.0	5	28.5
Cerebratulus lacteus (ribbon worm, Nemertea)	1	0.74	10.0	25	27.2
Chaetopterus variopedatus (tube worm, Annelida)	1	19.0	10.5	50	30.5
Callinectes sapidus (blue crab, Arthropoda)	Digestive gland: 1	3.34	6.0	32	27.2
Limulus polyphemus (horseshoe crab, Arthropoda)	Digestive gland: 1	44.0	20.0	50	27.6
Crassostrea virginica (oyster, Mollusca)	Mantle tissue: 22	20.0	100.0	120	28.0
Bugula neritina (bryozoan, Ectoprocta)	Many colonies	50.0	100.0	50	24.0
Lingula unguis (lampshell, Brachiopoda)	Soft parts: 6	3.84	9.5	25	24.5
Leptosynapta inhaerens (sea cucumber, Echinodermata)	25	13.0	6.0	32	27.0
Saccoglossus kowalevskii (acorn worm, Hemichordata)	Pieces: 6	2.01	6.0	32	26.0
Styela plicata (tunicate, Chordata)	6	24.0	24.5	25	28.0

Table 2. Percentage of radioactivity in organic acids after exposure of animals to $NaHC^{14}O_3$.

Species	Count/min*	Acids†								Total recovery
		Suc	Fum	Mal	Cit	Iso	αKg	Unkn	Lac	
Hymeniacidon heliophila	613	61.2	25.5	12.3	0		0		0	99.0
Aiptasia pallida	1101	52.0	3.4	10.1	7.1		3.5		0	76.1
Bdelloura candida	4275	4.0	0.8	10.4	0.2	0.4	0.3	65.5	1.1	82.7
Stylochus zebra	260	8.7	3.7	17.4	0.9	18.8	0	41.9	0.9	92.3
Cerebratulus lacteus	1123	79.7	0.5	27.4	0		0		0	107.6
Chaetopterus variopedatus	2305	60.8	9.3	12.6	0.7		1.2		9.6	94.2
Callinectes sapidus	1983	82.0	6.6	9.4	0.1		3.4		2.5	104.0
Limulus polyphemus	844	21.6	20.5	17.5	6.0		3.2	6.3	17.4	92.5
Crassostrea virginica	2633	99.0	0.7	1.4	0		0		0	101.1
Bugula neritina	658	53.5	8.0	2.0	0	0		0	0	63.5
Lingula unguis	1651	92.9	1.7	0.6	0	0	0		0.4	95.6
Leptosynapta inhaerens	1293	20.8	8.1	16.8	7.5	17.4	10.0	4.2	0.9	85.3
Saccoglossus kowalevskii	3399	66.0	4.8	12.2	0.4	0.4	0.3	5.0	1.5	90.6
Styela plicata	507	30.5	3.6	15.8	5.7	6.5	1.5	6.9	1.1	71.6

* Radioactivity of acetone extracts at origin of chromatograms. † Succinic, fumaric, malic, citric, isocitric, α-ketoglutaric, unknown, and lactic acids, respectively.

listed in Table 2. Twelve of the species examined proved to have the major portion of the radioactivity recovered in three acids: succinic, fumaric, and malic. The exceptions were the flatworms, which had most of the radioactivity in unidentified acids. In the sea cucumber the acids of the cycle were more uniformly labeled than in any of the other animals. Zeros in Table 2 indicate that no radioactivity was found, and blank spaces indicate that no assay was made. The total recovery is the sum of the percentages found in individual acids and was more than 63 percent in all cases. The lower recoveries were in experiments which presented difficulty in eliminating pigments from the acetone extracts. Nine species yielded labeled lactic acid in addition to acids of the Krebs cycle.

The results demonstrate that CO_2 fixation occurs in many invertebrate groups. Earlier studies had shown that representatives of five animal phyla—namely, protozoa, nematodes, insects, echinoderms, and vertebrates—fix CO_2 or have enzymes capable of fixation. This report establishes the occurrence of fixation in marine representatives of 12 phyla, including three among the five already known, making a total of 14 so far examined. There are, however, some 22 recognized phyla of animals, and a number of radically distinct subphyla and classes within some of them, so that in a taxonomic sense a large fraction of the animal kingdom has not yet been investigated.

The operation of the citric acid cycle in the tissues of numerous invertebrates has been inferred from the evidence of increases in oxygen consumption on supplying cycle intermediates and inhibition by specific inhibitors, and occasionally from demonstration of enzymatic activities intrinsic to the cycle. Some invertebrates for which such evidence has been presented include *Ascaridia galli* and three other species of parasitic nematodes (*7*), the rat tapeworm *Hymenolepis diminuta* (*8*), an oligochaete worm *Peloscolex velutinus* (*9*), and the snails *Australorbis glabratus* (*10*) and *Helix pomatia* (*11*). Rockstein (*12*) has reviewed demonstrations of Krebs cycle activity in a variety of insects, although the work of Chance and Sacktor (*13*) suggests that this pathway is not of primary importance in insect flight metabolism.

Marine invertebrates in which the Krebs cycle is presumed to be active, on the basis of respiratory stimulation, include eggs of *Echinus esculentus,* investigated by Cleland and Rothschild (*14*), and eggs of three other species of sea urchins in work reviewed by them. Similar demonstrations have been made with eggs of the oyster *Ostrea commercialis* (*15*) and mantle tissue of the oyster *Crassostrea virginica* (*16*). In some marine invertebrates, for example, the sponge *Microciona prolifera* (*17*) and the spiny lobster *Panulirus japonicus* (*18*), stimulation of oxygen uptake by the addition of cycle intermediates could not be demonstrated.

The presence of the acids themselves in tissues would also provide evidence in favor of the cycle, but they are rarely demonstrated because they are in general "present in animal tissues in minimal quantities, and do not accumulate to an appreciable extent," according to Fruton and Simmonds (*19*). In this study the finding of radioactivity can be taken as evidence of succinic, fumaric, and malic acids in tissues of all 14 species, and evidence of two or more other acids of the cycle in nine species. The large accumulation of radioactivity in succinic acid in most of the animals was also found in the oyster mantle, in which fixation is accomplished by the conversion of propionate to succinate. It is not at all unlikely that the same pathway could be shown in these species. If this conversion occurs, then endogenous propionic acid would be expected. Considerable quantities of propionic and acetic acids have been reported present in a marine tube worm, *Eudistylia vancouveri* (*20*). The experiments on the two flatworms suggest quite a different pathway of CO_2 fixation in this group, which will be the object of further study.

The quantitative significance of invertebrate CO_2 fixation in the carbon cycle of the sea remains to be assessed. If the process is as widespread among marine invertebrates in general as these results suggest, then the portion of their respiratory CO_2 which they withhold for synthetic purposes may be large enough to affect the supply available to the photosynthetic organisms upon which the productivity of the sea is based.

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References and Notes

1. C. S. Hammen and K. M. Wilbur, *J. Biol. Chem.* **234**, 1268 (1959).
2. T. Hultin and G. Wessel, *Exptl. Cell Research* **3**, 613 (1952).
3. This work was begun with the aid of a grant from the Office of Naval Research to K. M. Wilbur, and continued with support from the National Science Foundation to C. S. Hammen. One of us (P.J.O.) held a summer research fellowship of the American Physiological Society as a faculty member of Lynchburg College, Lynchburg, Va.
4. Specimens of *Lingula* were collected at Kaneohe, Hawaii, by Mrs. Dora May Banner, and shipped via air mail to Beaufort, N.C.
5. C. E. Frohman, J. M. Orten, A. H. Smith, *J. Biol. Chem.* **193**, 277 (1951).
6. J. Carles, A. Schneider, A. M. Lacoste, *Bull. soc. chim. biol.* **40**, 221 (1958).
7. V. Massey and W. P. Rogers, *Nature* **163**, 909 (1949).
8. J. W. Daugherty, *Proc. Soc. Exptl. Biol. Med.* **85**, 288 (1954).
9. D. Petrucci, *Riv. biol.* (*Perugia*) **46**, 241 (1954).
10. E. C. Weinbach, *Arch. Biochem. Biophys.* **42**, 231 (1953).
11. K. R. Rees, *Biochem. J.* **55**, 478 (1953).
12. M. Rockstein, *Ann. Rev. Entomol.* **2**, 19 (1957).
13. B. Chance and B. Sacktor, *Arch. Biochem. Biophys.* **76**, 509 (1958).
14. K. W. Cleland and Lord Rothschild, *J. Exptl. Biol.* **29**, 416 (1952).
15. K. W. Cleland, *Australian J. Exptl. Biol. Med. Sci.* **29**, 35 (1951).
16. L. H. Jodrey and K. M. Wilbur, *Biol. Bull.* **108**, 346 (1955).
17. E. E. Gordon, M. Spiegel, C. A. Villee, *J. Cellular Comp. Physiol.* **45**, 479 (1955).
18. B. T. Scheer, C. W. Schwabe, M. A. R. Scheer, *Physiol. Comparata et Oecol.* **2**, 327 (1952).
19. J. S. Fruton and S. Simmonds, *General Biochemistry* (Wiley, New York, ed. 2, 1958), p. 516.
20. D. R. Idler and U. H. M. Fagerlund, *Fisheries Research Board Can. Progr. Rept. No. 97* (1953), p. 6.

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