A SURVEY OF HAEM COMPOUNDS IN INVERTEBRATES USING THE BENZIDINE TEST

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INTRODUCTION

In spite of the fact that more than a quarter of a century has already elapsed since KEILIN (1925) published his fundamental work on cytochrome, the occurrence of this pigment in lower organisms has not been widely investigated as HUMPHREY (1947) pointed out. Cytochrome oxidase, on the contrary, has been found to be of frequent occurrence, although there is still much to be done in Invertebrates regarding this enzyme. HUMPHREY (1947, 1948) studied the oyster muscle and there is reason to believe (see WIERSMA 1952) that, in what concerns haem pigments acting on cellular respiration, the situation has not improved much since. Yet, the search for haem compounds in animals is of unquestionable importance and specially interesting in the approaches towards a better understanding of the relation between enzymatic equipment and degree of activity.

Haem compounds acting on cellular respiration are usually detected either by direct spectroscopic observation after crude or more elaborated extraction or by the ability of a tissue extract (or homogenate) to catalyse the air oxidation of reduced cytochrome. Among Invertebrates, however, the amount of such haem compounds can often be to small to be detected by spectroscopical methods and the measurement of oxidase activities by the current techniques may not be considered alone as a full evidence of the presence of haem compounds. The last resort is then the benzidine test. This test, for instance, can afford a further evidence of the presence of the succinic oxidase system in cases where despite the failure of detecting cytochrome spectroscopically (HUMPHREY 1947) the measurement of the enzymatic activity has shown it to exist. As a matter of fact, the benzidine test should be the first natural step in any study of biological systems possibily involving haem compounds.

The opportunity provided by frequent stays at the new Marine Biological Laboratory of São Sebastião and the vicinity of our laboratory in São Paulo to Institute Butantan led us to survey different marine and terrestrial Invertebrates for haem compounds using the benzidine test. The main scope of the survey was the study of the correlation between the intensity of the response to the test and the degree of activity of the animals. Our thanks are due to the Marine Biological Laboratory and to Institute Butantan for the facilities and material granted and to Drs. W. Bücherl, M. Vannucci and L. R. Tommasi for the classification of some of the animals.

METHODS AND RESULTS

Fresh benzidine was prepared by adding sodium acetate to benzidine hydrochloride. The reaction products were filtered in a Buchner and the deposited benzidine was washed with glacial acetic acid during the filtration. The cake obtained was kept saturated in glacial acetic acid. The test was applied as follows.

Equivalent amounts of total animals or excised parts were ground in a mortar and received 2 ml of benzidine solution plus drops of peroxide (10 vols.). In Arthropods, whenever it was impossible due to the small size of the animals, to excise the soft adhering structures from the chitinous squeleton, the test was applied as did PRENANT (1927) and more recently HANSON (1950), among others, to trace the course of blood vessels. The intensity of the response was arbitrarily classified as negative, scarce, fair and strong, as has been done previously (KEILIN 1925, HUMPHREY 1947), and the assumption was also made that the intensity of the response somewhat reflected the amount of haem compound present.

Table I shows the results obtained and is also an attempt to put together all the available data on the occurrence of haem compounds in Invertebrates. From the table are excluded data concerning haem compounds which function as blood oxygen carriers or storers, namely, hemoglobin, myoglobin, erythrocruorin and chlorocruorin, because they are already nicely arranged by PROSSER (1952, pp. 291-292, table 48).

As to our own results, the data of table I indicate that:

a) Red sponges exhibited a fair reaction to the test, whereas green and bluish species not only did not respond to the test but also exhibited the striking ability of bleaching the positive reaction given by a solution of pure cytochrome (**MENDES & KNAPP** 1956).

b) Planctonic medusae gave only scarce responses, whereas Anthozoans showed strong reactions. The Ctenophore did not respond to the test.

c) Terrestrial and marine Isopods exhibited responses from scarce to fair according to the body fragment used. This relation between body part and the degree of the response was still more marked in the case of the two crabs more intensively studied.

d) In the Scorpions and the Spider, the reaction was either negative or scarce for muscles of the movable appendages, but positive when the dorsal abdominal carapace (which included the adhering heart), the fatty abdominal content (spider) or eggs (Scorpion) were used. Scorpion embryos (total, prehatching strages) gave only a fair response.

e) The mantle and body wall muscles of the sea hare (*Aplysia*) strongly responded to the test, whereas total stomach and intestine exhibited a fair response. The oyster foot very definitely responded to the test.

f) The sea cucumber and the sea urchin gave either scarce responses (longitudinal and lantern muscles) or a negative test (intestine, ripe ovary and testis). In the latter case, the material was also able to reverse the positive benzidine test given by pure cytochrome, as in the case of green sponges. The sea lily (Crinoid) neither responded to the nor was able to reverse a positive benzidine test. Ripe ovary and testis of the 9 armed bluish *Luidia* behaved like the same organs of Holothurians and Sea Urchins, bleaching the positive test obtained with cytochrome. On the contrary, T A B L E I

The detection of haem compounds in some marine and terrestrial Invertebrates using the benzidine test.

Phylum	Group	Species	Organ	Part	Benz. Test	Reversal	Author
Spongiaria	?	red (unclassif.)	total	—	fair	—	Mendes & Knapp 1957
	?	green (")	"	—	negative	+	" "
Coelenterata	Limnomedusae	*Olindias sambaquiensis*	"	—	scarce	—	" "
	Trachymedusae	*Liriope tetraphylla*	"	—	"	—	" "
	Anthozoa	*Bunodactis* sp.	body wall	muscles	strong	—	" "
	"	*Palythoa* sp.	total	—	"	—	" "
	Ctenophora	*Mnemiopsis McGradyi*	"	—	negative	—	" "
Plathyhelminthes	Turbellaria	*Dendrocoela lactea*	?	?	positive (*)	—	Keilin 1925
Annelida	Oligochaeta	*Allolobophora chlorotica*	?	?	"	—	"
		Helodrilus caliginosus	?	?	"	—	"
Aschelminthes	Nematodes	*Ascaris megalocephala*	?	?	"	—	"
		Ascaris suis	?	?	"	—	"
Arthropoda	Xiphosura	*Limulus polyphemus*	heart	total	" (*)	—	Ball & Meyerhof 1940
			claw	yellow mm.	"	—	" "
			squelet.	" "	"	—	" "
			"	white "	negative	—	" "
			blood clot	fraction	positive	—	" "
			eggs	ground	"	—	" "
	Crustacea	*Asellus aquaticus*	?	?	"	—	Keilin 1925
		Oniscus sp.	?	?	"	—	"
		Armadillidium vulgare	thorax	muscles	fair	—	Mendes & Knapp 1957
			"	total	strong	—	" "
			leg	"	"	—	" "
			gut	"	"	—	" "

	Lygia exotica	"	"	scarce	—	" "
		leg	"	strong	—	" "
		thorax	muscles	"	—	" "
	Homarus americanus	heart	total	positive	—	Ball & Meyerhof 1940
		claw	muscles	"	—	" "
		squelet.	"	"	—	" "
	Cancer pagurus	?	?	"	—	Keilin 1925
	Grapsoid crab (unclass.)	chela	muscles	scarce	—	Mendes & Knapp 1957
	Callinectes sp.	"	"	"	—	" "
		walking leg	"		—	" "
		swimming leg	"	fair	—	" "
	Uca sp.	chela	muscles	negative	—	" "
		walking leg	"	scarce	—	" "
		heart	total	strong	—	" "
		scaphonathite	muscles	"	—	" "
Scorpionidea	*Tityus serrulatus*	tail	"	scarce	—	" "
		chele	"	negative	—	" "
		cephalothorax	total	strong	—	" "
	Tityus bahiensis	tail	muscles	scarce	—	" "
		chela	"	negative	—	" "
		body cavity	content	fair	—	" "
		ovary	eggs	strong	—	" "
		embryos	total	fair	—	" "
Araneida	*Epeira diademata*	?	?	positive	—	Keilin 1925
	Pamphobeteus roseus	leg	muscles	scarce	—	Mendes & Knapp 1957
		palp	"	"	—	" "
		cephalothorax	total	strong	—	" "
		abdomen	content	"	—	" "
Myriapoda	*Lithobius forficatus*	?	?	positive	—	Keilin 1925
	Geophilus sp.	?	?	"	—	"

Insecta		Several Diptera					
		” Hymenoptera					
		” Coleoptera	wing	brown mm.	strong	—	”
		” Lepidoptera	leg	white mm.	scarce	—	”
		” Hemiptera					
		” Orthoptera					
Mollusca	Gastropoda	*Helix*	radula	muscles	positive (*)	—	Baldwin 1938
			”	”	” (*)	—	Dhéré & Vegez **
		Helix nemoralis & *aspersa*	?	?	”	—	Keilin 1925
		Limnea peregra & *stagnalis*	?	?	”	—	”
		Busycon canaliculatum	radula	muscles	” (*)	—	Dakin **
			”	”	” (*)	—	Mendel & Bradley **
			”	”	”	—	Ball & Meyerhof 1940
			heart	total	”	—	” ”
		Aplysia sp.	mantle	muscles	strong	—	Mendes & Knapp 1957
			body wall	”	”	—	” ”
			stomach	total	fair	—	” ”
			intestine	”	”	—	” ”
	Pelecypoda	*Ostrea edulis*	tissues	various	” (*)	—	McMunn **
		Ostrea sp.	foot	muscles	strong	—	Mendes & Knapp 1957
		Saxostrea commercialis	foot	muscles	positive (*)	—	Humphrey 1948
		Venus mercenaria	heart	total	”	—	Ball & Meyerhof 1940
			valves	adductor m.	”	—	” ”
	Cephalopoda	*Loligo pealii*	heart	total	”	—	” ”
			head	retractor m.	”	—	” ”
			neck	” ”	”	—	” ”
Echinodermata	Holothuroidea	Holothuria grisea	body wall	long. mm.	scarce	—	Mendes & Knapp 1957
			intestine	total	negative	+	” ”
			ovary	”	”	+	” ”
			testis	”	”	+	” ”

	Echinoidea	Arbacia punctulata	testis	sperm	positive	—	Krahl et alt. 1941
			"	"	positive	—	Ball & Meyerhof 1940
			ovary	eggs	"	—	" "
		Echinometra locunter	lantern	muscles	scarce	—	Mendes & Knapp 1957
			testis	total	negative	+	" "
			ovary	"	"	+	" "
	Crinoidea	Tropiometra car.	body	"	"	—	" "
	Asteroidea	Luidia senegalensis	testis	"	"	+	" "
			ovary	"	"	+	" "
		Echinaster	testis	"	strong	—	" "
			ovary	"	"	—	" "
			body	fluid	"	—	" "
			arms	muscles	"	—	" "
	Ophiuroidea	Ophionereis sp.	disc	total	"	—	" "
			arms	"	"	—	" "
Tunicata	Ascidiacea	Molgula manhattensis	intestines	"	scarce	—	Ball & Meyerhof 1940
		Phallusia mammilata	testis	sperm	positive (*)	—	Califano & Boeri 1950
			ovary	ripe eggs	"	—	" "
		Phallusia nigra	blood	fluid	negative	+	Mendes & Knapp 1957
Acrania	Leptocardia	Branchiostoma platae	body wall	total	strong	—	" "

* Implication from spectroscopic and enzyme measurements of cytochrome and cytochrome oxidase.

** Authors not mentioned in the literature, cited *apud* Ball & Meyerhof 1940.

the red, 5 armed starfish (*Echinaster*) exhibited ripe ovary and testis and arm muscles with strong benzidine reactions. Finally, the brittle star (Ophiuran) was, among Echinoderms, the one who really gave a remarkable response to the test (as strong as those obtained with cytochrome, Vertebrate blood or Insect wing muscle).

g) The body fluid of the Ascidia was very active in reversing the positive benzidine test obtained with cytochrome. The body wall of the Acranian *Branchiostoma* gave a strong response to the test.

DISCUSSION

Although the main scope of the present survey was to study the relation between the intensity of the response to the benzidine test and the degree of activity of the animal (or part thereof), the results obtained serve also as a starting point to further research of the cellular respiratory enzymes of those animals which definitely gave a positive reaction. For a great majority of the cases gathered in Table I from previous authors this has already been done partially or totally. As a matter of fact, the word "positive" for the benzidine test in cases such as KEILIN's of BALL & MEYERHOF's experiments is a mere implication from spectroscopic and enzyme measurements of cytochrome and cytochrome oxidase, the authors (except for KEILIN's Insect data) actually having not reported results of benzidine tests. The data of Table I suggest the following comments.

Sponges are relatively inactive animals, with no nerve cells. The spindle shaped muscles which bring about the closure of the oscula have been called independent effectors (PARKER 1917) because they combine sensory and motor functions. The fair benzidine test obtained with red species indicates either a small amount of cytochrome (or cytochrome oxidase) or, at least, the hemochromogen precursor mentioned by KEILIN (1929). The negative response given by green sponges does not necessarily mean the absence of haem compounds, since as previously reported (MENDES & KNAPP 1. c.), the reversal of the benzidine test obtained with pure cytochrome after addition of ground green sponges suggests the presence of a compound (possibily analogous to the vanadium chromogen of certain Ascidians) which masks the presence of haem compounds when the test is employed. A further consideration of this point will be made below in the discussion of the results obtained with Holothurians and Ascidians.

The scarce response given by planctonic medusae might be interpreted as a discrepancy between the degree of activity of these lively pulsating organisms and the amount of possibily occurring haem compounds involved in the energy producing cycles. Their strikingly high water content, however, may have influenced the response, the amount of non aqueous material actually used in the tests being much smaller than in other cases. The actinian (*Bunodactis*) and the colonial form *Palythoa* responded according to their well developed muscular system which enables the animals to quickly react to stimulation. The Ctenophore (*Mnemiopsis*) is a feeble swimmer, slowly moving by ciliary action. It also possesses a remarkably high water content. The negative response could then be explained on the basis of these two factors.

The results obtained with Crustaceans call to mind BALL & MEYERHOF's puzzle before the whelk (*Busycon*) and the lobster (*Homarus*): Why should an animal employ hemocyanin for a blood pigment and yet possess muscles rich in myoglobin (*Busycon*) or other haem compounds such as cytochrome and cytochrome oxidase? They did not really attempt to solve this puzzle, nor did HUMPHREY (1. c.) in his oyster's study. BALL & MEYERHOF, however, tried to correlate the occurrence of hemoglobin and hemocyanin with the presence of blood cells. The greater molecular size of hemocyanin would make unnecessary its inclusion in special blood cells. The same would happen to the Invertebrate hemoglobins ("erythrocruorins" ROCHE 1934), of comparable molecular size, which occur in the cell free blood of certain worms. They also stated that hemoglobin, when it does appear as an oxygen carrier, is contained in special blood cells. This view, of course, is supported by the admission that (a) the greater molecular size of extracellular hemoglobins serve to confine the molecules to the circulatory system (PROSSER 1952), (b) hemoglobins in solution can only exist in low concentrations because otherwise it would cause either a high degree of viscosity (larger molecules) or a high coloidal osmotic pressure (smaller molecules). Besides, within the blood cells haemoglobins would have the proper chemical environment which may be of some funccional significance (BARCROFT 1922). As well known, this admission has contributed to form the controverted opinion that Invertebrate hemoglobins do not function normally as carriers, merely storing oxygen for eventual exposures to low tensions of this gas. We think that a more plausible explanation of BALL & MEYERHOF's puzzle can be offered without appealing to such a controverted argument. The data of Table I for animals possessing hemocyanin, specially our Isopod and Decapod series, indicate that the response to the benzidine test invariably increased when we passed from little active to rhythmically operating structures. This is beautifully shown in the case of the fiddle crab (*Uca*): chela, walking leg, heart and scaphognathite. BALL & MEYERHOF also noticed that in the squid, the most active animal examined, the head and neck retractors have low concentrations of haem compounds as compared with main heart ("the ideal material for demonstrating the cytochrome spectra") and that in the lobster the heart is rich in cytochrome and cytochrome oxidase, whereas the claw and squeleton muscles are poor. Thus, it seems that although using hemocyanin as a blood oxygen carrier, these organisms make use of a fitter intracellular oxidase system (cytochrome and cytochrome oxidase) or oxygen storer (myoglobin) whenever a higher degree of activity is required. We must finally bear in mind that the presence of haem compounds in animals which use hemocyanin as blood oxygen carrier looses much of its puzzling character when consider the widespread occurrence of heme in animals. In fact, it has been utilized in blood or intracellular pigments in a wide variety of unrelated animals regardless of the system transporting oxigen to the tissues (KEILIN 1925, KROGH 1941, PROSSER l. c.).

The strong response obtained with Spider's or Scorpion's more active organs (such as the abdominal dorsal carapace — which included the heart — and the eggs) and the fair test showed by less active parts (such as the muscles of the movable appendages and late embryos) might be explained along the same line of reasoning. The same would also apply to *Aplysia's* data: the strong response of mantle and body wall muscles and the fair test of the stomach and intestine. It is not easy, however, to explain the strong response of the fatty content of the spider.

A nice correlation between activity and presence of haem compounds might have been ascribed to Echinoderms, had we not observed that in most cases where the benzidine test was negative the material was also able to reverse the positive test given by pure cytochrome. In fact, judging by the response alone to the test, one might say that Holothurians, one of JORDAN's (1914) "reflexarme Tiere", and the little active Echinoids and Crinoids exhibited accordingly a negative reaction, whereas the more lively Asteroids and the quite active Ophiuroids strongly reacted to the test. We are not entitled, however, by the Benzidine test alone, to say that Holothurian or sea urchin eggs and sperm do not possess haem compounds, since their occurrence may have been masked during the test by the presence of that compound which is responsible for the observed reversal of the benzidine reaction, as already mentioned. Even in the active bluish starfish *Luidia* the benzidine test was unable to reveal heme derivatives probably in consequence of such a reversing agent. On the other hand, Holothurian longitudinal muscles and sea urchin lantern muscles did respond, although scarcely, to the test and BALL &MEYERHOF (l.c.) reported the presence of cytochrome and cytochrome oxidase in *Arbacia* sperm. They failed, howewer, to detect these haem compounds in the eggs, not even after fertilization (in one experiment with KRAHL). Thus, the situation in the sea urchin seems rather complex. The fact that by methods other than the benzidine test no detectable amount of haem compounds could be found in Arbacia eggs might indicate that if any such compound is really present it cannot be of much functional importance. KRAHL, KELTCH, NEUBECK & GLOWES (1941) also worried about this question and said that "it seems safe to conclude that cytochrome C cannot carry a significant fraction of the oxygen consumption". It remains finally the possibility that the compound responsible for the reversal of the benzidine test could have also acted as an inhibitor in the methods used by BALL & MEYERHOF with *Arbacia* eggs.

The blood of the sessile *Ascidia nigra* strongly bleaches the benzidine test given by a solution of pure cytochrome. Here and in a previous paper (MENDES & KNAPP, l. c.) we have been attempting to correlate this finding (as well as identical results obtained with bluish green sponges and the gonads of Holothurians, sea urchins and of the bluish starfish *Luidia*) with the presence of vanadium, which has already been proved to occur in Ascidians (HENZE 1911) and Holothurians (PHILIPPS 1914). Recently, CALIFANO & BOERI (1950) showed that in the Ascidian *Phallusia mammilata,* the vanadium compound ("hemovanadin") reduces cytochrome both under aerobic and anaerobic conditions. They did not imply, however, that its function is necessarily connected with that of the carrier. The fact that the sperm and ripe eggs of *P. mammilata* exhibit cytochrome c at the spectroscopic analysis raises the interesting question of the compatibility of cytochrome c with such a strong reducing agent within the same organism, which deserves further consideration. BALL & MEYERHOF (l. c.) also noticed the presence of Vanadium in the blood and haem compounds in the tissues of the small Ascidian *Molgula manhattensis*. The fact that they had trouble with the spectroscopic determination of the three cytochromes and the test of the succinodehydrogenase activity may be linked to an interfering action of the vanadium compound.

SUMMARY

1. Several terrestrial and marine Invertebrates were surveyed for haem compounds through the benzidine test, with the main scope of studying the correlation between the intensity of the response to the test and the degree of activity of the animals (or parts therof).

2. Table I shows the results obtained and is also an attempt to put together all the available data on haem compounds in Invertebrates, with the exclusion of those involved in blood transport or tissue storage of oxygen.

3. Using the intensity of the response as a semi-quantitative criterium of the presence of haem compounds a well established correlation with activity could be obtained in cases such as An-

thozoans, Ctenophores, Decapod Crustaceans, red starfishes, Ophiuroids and the Acranian.

4. BALL & MEYERHOF's puzzle concerning the concomitant presence of hemocyanin as a blood carrier and haem compounds as tissue oxygen storer or carrier is discussed in view or the results obtained with Crustaceans, Scorpions, Spiders and Molluscs.

5. The striking ability of some Invertebrate tissues (total green or bluish sponges, the gonads of Holothurians, sea urchins and the bluish starfish *Luidia* and the blood of the Ascidian) to bleach the positive benzidine test given by pure cytochrome (MENDES & KNAPP 1956) is focused in terms of its possible interference with the processes of detecting haem compounds.

SUMÁRIO

1. Diversos invertebrados terrestres e marinhos foram pesquisados pelo teste da benzidina em busca de compostos hêmicos, com a finalidade de estudar a correlação entre a intensidade da resposta e o grau de atividade do animal (ou de suas partes).

2. A Tabela I mostra os resultados obtidos e é também uma tentativa de agrupar os dados disponíveis sôbre os compostos hêmicos em Invertebrados, exclusive os empenhados no transporte sangüíneo ou no armazenamento tissular de oxigênio.

3. Usando-se a intensidade da resposta como critério semiquantitativo da presença de compostos hêmicos, pôde ser obtida uma bem estabelecida correlação com a atividade em casos tais como Antozôos, Ctenóforos, Crustáceos Decápodos, estrêlas do mar vermelhas, Ofiuros e o Acranio.

4. O enigma de BALL K MEYERHOF, relativo à concomitante presença de hemocianina como transportador sangüíneo e compostos hêmicos como armazenadores ou transportadores tissulares de oxigênio, é discutido à vista dos resultados obtidos com crustáceos, escorpiões, aranhas e moluscos.

5. A surpreendente capacidade que alguns tecidos de invertebrados (esponjas verdes ou azuladas totais; gônadas de holoturias, ouriços do mar e da estrêla azulada *Luidia;* sangue da ascídia) possuem de descorar (reverter) o teste da benzidina positivo dado por

citocromo puro é também focalizada em têrmos de sua possível interferência com os processos de evidenciar a existência de compostos hêmicos.

LITERATURE

BALDWIN, E. 1938. — On the respiratory metabolism of *Helix pomatia*. Biochem. J., v. 32, pp. 1225-1237.

BALL, E. G. & B. MEYERHOF, 1940 — On the occurrence of iron-porphirin compounds and succinic dehydrogenase in marine organisms possessing the copper blood pigment hemocyanin. J. Biol. Chem., v. 134, pp. 483-493.

BARCROFT, J. 1922 — The "raison d'être" of the red corpuscle. Harvey Lect., v. 17, pp. 146-161.

CALIFANO, L. & E. BOERI, 1950 — Studies on haemovanadin. III. Some physiological properties of haemovanadin, the Vanadium compound of the blood of *Phallusia mamillata*. J. Exp. Biol., v. 27, pp. 253-256.

HANSON, J., 1950 — The blood system in the Serpulimorpha (Annelida, Polychaeta). I. The anatomy of the blood system in the Serpulidae. Quart. J. Micr. Sci., v. 91, pp. 111-129.

HENZE, M., 1911 — Untersuchungen über das Blut des Ascidien. I. Die Vanadium — verbindung der Blutkörperchen. Hoppe-Seyl. Z. physiol. Chem., v. 72, pp. 494-501.

HUMPHREY, G. E., 1947 — The succinoxidase system in oyster muscle. J. Exp. Biol., v. 24, pp. 352-360.

—— 1948 — The effect of narcotics in the endogenous respiration and succinate oxidation in oyster muscle. J. Mar. Biol. Ass. Plymouth, v. 27, pp. 504-512.

JORDAN, H. 1914 — Über "reflexarme" Tiere. IV. Holothurien. Erste Mittel. Die Holothurien als hohlorganartige Tiere und die Tonusfunktion ihrer Muskulatur. Zool. Jahrb. Allg. Zool. u. Physiol., v. 34, pp. 365-436.

KEILIN, D. 1925 — On cytochrome, a respiratory pigment common to animals, yeast and higher plants. Proc. Roy. Soc. London, B, v. 98, pp. 312-339.

—— 1929 — Cytochrome and respiratory enzymes. Proc. Roy. Soc. London, B., v. 104, pp. 206-252.

KRAHL, M. E., A. K. KELTCH, C. E. NEUBECK & C. H. A. CLOWES, 1941 — Studies on cell metabolism and cell division. V. Cytochrome oxidase activity in the eggs of *Arbacia punctulata*. J. Gener. Physiol., v. 24, pp. 597-617.

KROGH, A. 1941 — The Comparative Physiology of Respiratory mechanisms. VII + 172 pp. Univ. Philadelphia Press.

MENDES, E. G. & E. P. KNAPP. 1956 — Reversão do test de benzidina por material marinho. Ciência e Cultura, v. 8, pp. 170-171.

PARKER, H. G. 1919 — The Elementary Nervous System. Lippincott, Philadelphia, 229 pp.

PHILLIPS, A. H. 1918 — A possible source of Vanadium in sedimentary rocks. Amer. Journal Sci. v. 46, pp. 473.

PRENANT, M. 1921 — Sur une technique de coloration des vaisseaux. Bull. Soc. Zool. France, v. 46, pp. 140-143.

PROSSER, C. L. 1952 — Comparative Animal Physiology. IX + 888 pp. Saunders. Philadelphia.

ROCHE, J. 1934 — Introduction à la biochimie comparée des pigments respiratoires. Ann. Physiol. et Physicochem., v. 10, pp. 583-591.

WIERSMA, C. A. 1952 — Comparative physiology of invertebrate muscle. Ann. Rev. Physiol., v. 14, pp. 159-176.