Tabelle I

Verteilung der Oxydationsenzyme im Seeigelei (*Paracentrotus lividus*)

Versuch Nr.	Zellfraktion	Zytochrom-oxydase	DPNH-Zytochrom-c-Reduktase	Succinat-Zytochrom-c-System
VII	$Ü_0$	3,44	5,55	0,68
VIIIa	Mitochondrien	3,18	3,04	0,65
(unbefruchtet)	Mikrosomen	0	2,21	0
	Überstand		0,12	
	$Ü_0$		6,93	0,42
X	Mitochondrien	3,18	3,18	0,16
(unbefruchtet)	Mikrosomen	0	2,74	0
	Überstand		0,3	
	$Ü_0$		4,49	0,51
VIIIb	Mitochondrien		2,17	0,14
(befruchtet)	Mikrosomen		1,98	0
	Überstand		0,09	

Gewinnung und Befruchtung der Eier erfolgte nach üblichen Methoden. Die Eier wurden nach [9] mit der Modifikation gewaschen und homogenisiert, dass im Homogenisierungsmedium Glyzylglyzin durch Phosphat ersetzt wurde. Die überstehende Suspension $Ü_0$ wurde durch Zentrifugieren des Homogenates bei 1000 *g* (20 min; Abtrennung der nicht zerstörten Eier) gewonnen. Die Herstellung der Mitochondrien erfolgte bei 13000 g (90 min) und der Mikrosomen bei 80000 *g* (300 *min*). Die überstehende Lösung dieser Fraktion wird als «Überstand» bezeichnet. Die Messung der Enzymaktivität wurde nach [10] vorgenommen. Die Werte geben die Mikromole Zytochrom c an, die von den einzelnen Enzymen entweder oxydiert oder reduziert wurden, bezogen auf 1 g Ei-Frischgewicht. Untersuchungsmonate waren Oktober und November 1957.

Tabelle II

Hemmung der Mitochondrien-Enzyme durch RÜ-Hemmstoff und Einfluss von RÜ auf Mikrosomen

Fraktion	System	Aktivität		Hemmung %
		Kontrolle	+ RÜ	
Mitochondrien . .	Succ-Zyt.-c	11	0	100
	DPNH-Zyt.-c	34	11	68
Mikrosomen . . .	DPNH-Zyt.-c	29	30	—

Zahlen geben $\Delta E \cdot 10^3$/min im Ansatz an. Die Messung erfolgte nach[11].

Folgende weitere Befunde scheinen von Interesse:

1. Das Verhältnis Succinat zu DPNH-Enzymaktivität entspricht annähernd dem in der Rattenleber. Die leichte Zerstörbarkeit der Succinodehydraseaktivität, die bei der Fraktionierung beobachtet wird (siehe Tabelle I, Versuch X und VIII B), erklärt vermutlich frühere negative Befunde.
2. Die Diaphoraseaktivität ist verhältnismässig schwach, verglichen mit den Verhältnissen bei Säugetieren.
3. Bei Befruchtung sind auffallende Veränderungen weder bezüglich der Aktivität noch der Verteilung der untersuchten Atmungsenzyme gefunden worden.

Die hohe Aktivität sowohl der Zytochrom-Oxydase als auch der DPNH-Zytochrom-Reduktase-Systeme ergeben eine hohe Kapazität für den Wasserstoff- und Elektronen-Transport. Diese Ergebnisse sprechen nicht für einen enzymatischen Block oder eine Lücke[1] im Atmungssystem des unbefruchteten Seeigeleies.

S. RAPOPORT, E. C. G. HOFMANN
und ANNA GHIRETTI-MAGALDI

Physiologisch-chemisches Institut der Humboldt-Universität Berlin und Physiologische Abteilung der Zoologischen Station Neapel, 5. Februar 1958.

Riassunto

Le attività citocroma ossidasica, succino-deidrogenasica, DPNH e TPNH cit. c riduttasica sono state studiate in omogenati e frazioni mitocondriali e microsomiali di uova vergini e fecondate di *Paracentrotus lividus*. I mitocondri contengono tutta l'attività citocromossidasica e succinodeidrogenasica, mentre i microsomi solo una metà della attività DPNH cit. c riduttasica totale. Spettri differenziali hanno rivelato la presenza, nei microsomi, di un citocromo avente una banda γ a 428–430 mμ.

A Cytochrome c from the Hepatopancreas of *Sepia officinalis* L.*

In the course of a recent investigation[1] on the respiratory enzymes in mitochondria isolated from several organs of cephalopods, direct proof of the presence of cytochrome c was not obtained. However, the difference spectra and the enzymatic activities of the particles indicated that the pigment was present, even if not easily detectable.

Having by chance a large number of living sepias in the laboratory suggested to us to attempt the extraction and purification of cytochrome c from different organs. The hepatopancreas was found to be the most suitable material. A total weight of 22 kg tissue was obtained and stored at – 20°C until needed.

Acid extraction and purification according to KEILIN and HARTREE[2] resulted in a complete loss of the pigment. Since all attempts based on acid treatment gave negative results, a method based on alkaline extraction was tried and found to be successful. The purification procedure of cytochrome c from sepia hepatopancreas was as follows:

Step 1.—2 kg of minced tissue were homogenized in Waring blendor for 2 min with 3 l of H_2O made alkaline with ammonia (pH ca. 8) and the suspension was centrifuged at 600 × *g* for 30 min. The upper layer (fat) was removed by suction, the supernatant stored in the cold and the sediment re-homogenized in 1 l of alkaline water and centrifuged as above. The combined supernatants were measured and to each 1100 ml, 100 ml of 25% lead acetate were added. The heavy precipitate was discarded by centrifugation at 2000 × *g* for 30 min and the supernatant was clarified by filtration on Hyflo supercell. The deep yellow-coloured solution was brought to pH 6 with

* This work has been supported by a grant (RG-4548) of the U.S. Public Health Service.

[9] M. E. KRAHL, A. K. KELTCH, C. E. NEUBECK und G. H. A. CLOWES, J. gen. Physiol. *24*, 597 (1941).

[10] S. RAPOPORT und CH. NIERADT, Hoppe-Seylers Z. *302*, 156 (1955).

[11] C. WAGENKNECHT und S. RAPOPORT, Hoppe-Seylers Z. *308*, 127 (1957).

[1] A. GHIRETTI-MAGALDI, A. GIUDITTA, and F. GHIRETTI, Biochem. J. *66*, 303 (1957); J. cell. comp. Physiol. (in the press).

[2] D. KEILIN and E. F. HARTREE, Proc. Roy. Soc. London *122B*, 298 (1937).

2 *N* acetic acid and to each 900 ml, 100 ml saturated sodium sulphate were added. The white precipitate of lead sulphate was discarded by centrifugation and the pH of the solution adjusted to 7·4 with 10% ammonia.

Step 2.—Solid $AmSO_4$ was added slowly until 75% saturation while the pH was maintained at 7·4. After 12 h in the cold, the precipitate was collected by filtration on Hyflo supercell, washed and dissolved in about 500 ml alkaline water (pH 7·4). The deep brown opalescent solution obtained was dialyzed against neutral $AmSO_4$ 50% saturation for 2–3 days. The precipitate was discarded by filtration and the brown-coloured fluid dialyzed against 75% saturated $AmSO_4$. The new precipitate formed was collected by filtration and dissolved in ca. 500 ml alkaline water. This treatment was repeated twice and the final solution (300 ml ca.) containing the fraction which precipitates between 50–75% saturated $AmSO_4$ was dialyzed overnight against running tap water.

Step 3.—The small precipitate formed was removed by centrifugation and the brownish-yellow-coloured supernatant was brought to pH 6 with acetic acid, then treated with phosphate gel (1 g dry weight per ml ca.). 10 ml phosphate gel were added to each 100 ml solution. The gel was removed immediately by centrifugation and the pinkish-yellow-coloured supernatant adjusted to pH 7·4. Saturated $AmSO_4$ was added until 70% saturation and the precipitate was centrifuged down and dissolved in 100 ml H_2O. The pink-yellow-coloured solution was dialyzed overnight against H_2O, treated again with phosphate gel and then with $AmSO_4$ as above. The precipitate was dissolved in 50 ml 0·01 *M* sodium alkaline phosphate and dialyzed against H_2O.

Step 4.—To the solution, solid $AmSO_4$ was added until 50% saturation. The yellow precipitate was discarded and the supernatant was brought to 70% saturation $AmSO_4$. The precipitate was collected and dissolved in the minimum volume (15 ml ca.) of 0·01 *M* sodium di-phosphate.

Step 5.—The yellow-red solution was centrifuged for 2 h at 70,000 × *g* in the Spinco preparative centrifuge and the small sediment discarded. The clear supernatant was then centrifuged again for 7 h at 105,000 × *g*. The top layer (yellow solution) was carefully pipetted and discarded, whereas the reddish middle part and the bottom sediment formed by almost pure cytochrome c were collected and used for all subsequent analysis.

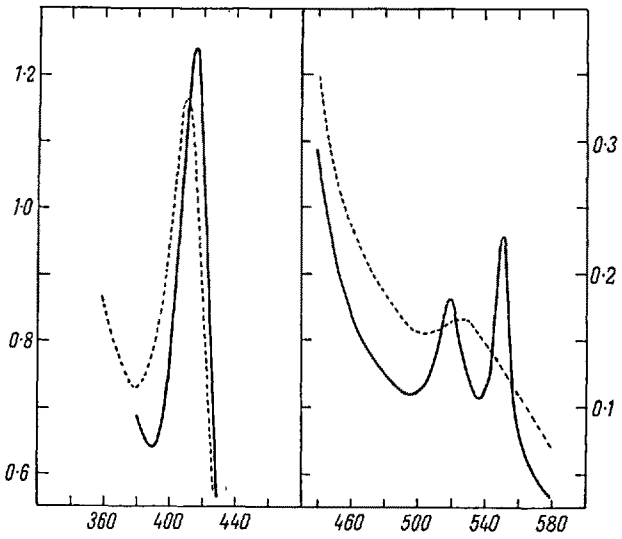
The spectrum of the pigment was taken at each step of purification procedure and the O.D. ratios at different wave lengths were compared with those of pure beef heart cytochrome c (Table). The final preparation contained 40 mg of protein per ml and 0·05% of iron as determined colorimetrically[3]. On paper electrophoresis (Whatman No. 1, 0·05 *M* phosphate buffer pH 7·3 or Veronal buffer pH 9) it showed one major and two minor components very slowly moving when compared with pure beef heart cytochrome c. Further purification by preparative paper electrophoresis in different conditions did not succeed because of the large adsorption when thicker paper was used. Elution of the paper also gave negative results.

Cytochrome c from Sepia's hepatopancreas in the oxidized form has maxima at 408 and 530 mμ; after reduction with sodium dithionite, bands with maxima at 416, 520 and 550 mμ appear (Fig.). Like mammal cytochrome c, this pigment does not form a pyridin haemochromogen at alkaline pHs and the prosthetic group is not split and dissolved by acidic acetone. Treatment with silver sulfate

O. D. Ratios during the purification procedure of Cytochrome c from Sepia hepatopancreas

	416 red / 550 red	416 red / 380 red	416 red / 408 ox	550 red / 536 red	550 red / 520 red	410 ox / 500 ox
Step 2, 1st ppt.	4·06	1·29	1·01	1·11	0·87	2·33
Step 2, 2nd ppt, dialy	4·72	1·23	1·00	1·30	0·94	4·11
Step 3	4·40	1·74	1·04	1·76	1·06	5·94
Step 4	4·74	1·80	1·06	1·77	1·19	5·70
Step 5, 1st centr.	5·46	1·81	1·04	2·08	1·28	7·09
Step 5, 2nd centr.	5·43	1·94	1·07	2·17	1·30	7·50
Beef Heart Cyt. c	4·60	6·04	1·25	3·53	1·71	13·97

according to PAUL *et al.*[4], followed by acidic acetone extraction, gives a product with an evident band at 391 mμ. The maximum at 625 mμ could not be detected owing to the high dilution of the initial preparation. Identical results were obtained, in the same conditions, with pure mammal cytochrome c.



Cytochrome c from the Hepatopancreas of *Sepia officinalis*. Spectrum of the oxidized form (dotted line) and after reduction with Sodium dithionite (full line). Abscissa wave length. Ordinate O.D.

Paper electrophoresis experiments were made with several buffers at pHs from 7·3 to 9·5. Like mammal cytochrome c, the pigment extracted from sepia hepatopancreas moves towards the negative electrode, an indication that the isoelectric points of both pigments have a very close value. Sepia's cytochrome c is completely reduced with ascorbate and rapidly oxidized with ferricyanide.

However, the cytochrome c extracted from sepia has several peculiar properties. It precipitates between 50 and 70% saturation $AmSO_4$ and is sedimented by prolonged centrifugation at 105,000 × *g*. After at 80°C for 2 min about 70% of the pigment is lost; destruction of the cytochrome occurs also at pH below 5. No change occurs when the spectrum is taken at different pHs from 5·5 to 9·0. The cytochrome is still partially reduced in an alkaline medium and the spectrum of the completely oxidized form can be obtained only in the precence of ferricyanide.

[3] H. THEORELL, M. BEZNAK, R. K. BONNICHSEN, K. G. PAUL, and A. AKESON, Acta chem. scand. *5*, 445 (1951).

[4] K. G. PAUL, Acta chem. scand. *4*, 239 (1950).

It is not oxidized by mammal cytochrome oxidase preparation[5] as demonstrated by manometric and spectrophotometric experiments.

ANNA GHIRETTI-MAGALDI and F. GHIRETTI

Department of Physiology, Stazione Zoologica, Naples (Italy), February 10, 1958.

Riassunto

Una emoproteina è stata estratta e parzialmente purificata dall'epatopancreas di *Sepia officinalis*. Essa è ossidata dal ferricianuro e ridotta dall'acido ascorbico; gli spettri di assorbimento delle due forme, ossidata e ridotta, sono quelli tipici del citocromo c di mammifero.

In comune con quest'ultimo il citocromo estratto dall'epatopancreas di sepia ha diverse altre proprietà come per esempio quella di non dare un emocromogeno con la piridina e di non perdere il gruppo prostetico dopo trattamento con acetone acido. A differenza del citocromo c di mammifero, il pigmento di sepia non resiste al calore e agli acidi; è precipitato dal solfato di ammonio al 75% di saturazione e sedimenta dopo prolungata centrifugazione a 105000 × *g*. Non è ossidato dalla citocromossidasi di mammifero.

[5] E. F. HARTREE, in K. PEACH and M. V. TRACEY, Modern Methods of Plant Analysis *4*, 197 (1955).

The Effect of some Common Fixatives on the Enzymatic Activity of Ribonuclease

While studying the action of ribonuclease on living oocytes of starfish, it was found that after freeze substitution basophily disappeared from the cytoplasm even though the oocytes were treated with ribonuclease only for 5 min. On the other hand, there was no loss in basophily when they were fixed in Zenker. These results are similar to what BRACHET[1] observed in the case of onion root tips. In later experiments, it was observed that the enzymatic activity of ribonuclease was not destroyed by freeze substitution. (A detailed account of this work will be published later.)

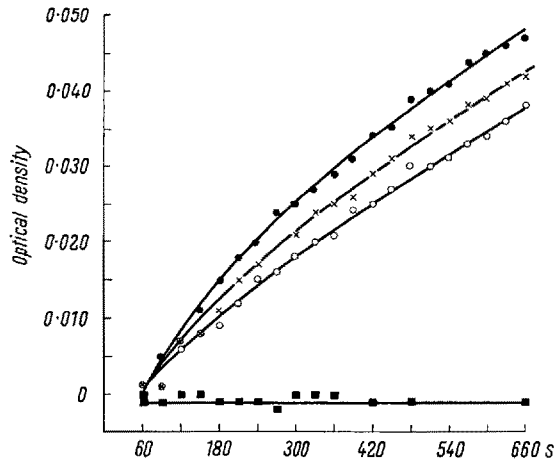
GHOSH[2] has reported that after freezing and thawing there is no loss in activity of DNase, as determined by its capacity to reduce Feulgen stainability. LAGERSTEDT[3] has suggested that RNase activity might be present in sections of pancrease fixed in Carnoy's fluid, and, after the addition of McIlvaine's Buffer it may initiate RNA digestion.

When this paper was under preparation, JONSSON and LAGERSTEDT[4] reported that RNase activity was retained in the sections of pancreas fixed in Carnoy, while measurable RNase activity could not be demonstrated in the formaldehyde fixed material.

The present work was undertaken with a view to ascertaining the effects of some common fixatives like alcohol–acetic acid (3:1), Serra and Zenker.

Methods.—Four tubes were taken and into each 2 ml of RNase (Armour Crystalline, 2 mg/ml) were pipetted. To the first tube was added 1 ml of alcohol-acetic acid, to the second 1 ml of Serra, to the third 1 ml of Zenker and to the fourth 1 ml of distilled water. After thorough mixing, the respective mixtures were allowed to stand for 1 h at room temperature (22°C). The mixtures were then dialysed in separate cellophane bags against distilled water for 24 h. When Zenker was added to the RNase solution, the latter became cloudy, probably due to the precipitation of RNase by the mercuric salts present in the fixative.

After the dialysis, the contents of the bags were centrifuged, and the supernatant in each was adjusted to equal volumes with distilled water. The enzymatic activity of these solutions was determined according to the method of KUNITZ[5].



Enzymatic activity of the solutions after dialysis.
• Control × Alcohol-acetic acid ○ Serra ■ Zenker

It is evident from the results obtained (Figure) that only the solution which was mixed with Zenker lost all its enzymatic activity, while the rest retained almost all of it. The RNase activity per minute calculated for each of these solutions is of the value of 0·006 for the control, 0·005 for the solution mixed with alcohol-acetic acid and 0·004 for the one mixed with Serra.

According to JONSSON and LAGERSTEDT, formaldehyde-fixation destroys the enzymatic activity of RNase. The results obtained by me show that the fixative, Serra, though it contains formalin, does not inactivate RNase. This may be due to the fact that the inactivation of RNase by formalin is reversible on dialysis.

These results indicate that RNase does not lose its enzymatic property after fixation in certain fixatives, and when these fixatives are completely removed during the process of washing and dehydration, the enzyme can digest the RNA of the sections after hydration of them.

Acknowledgment.—I wish to express my grateful thanks to Prof. J. BRACHET for suggesting the lines of investigation, his unfailing guidance and constant help, and also for the facilities afforded to me in his laboratory. My thanks are also due to Mme. Y. THOMAS for her generous help.

K. K. NAIR*

Laboratoire de Morphologie animale, Université libre de Bruxelles (Belgique), le 28 novembre 1957.

[1] J. BRACHET, Biochem. biophys. Acta *19*, 583 (1956).
[2] C. GHOSH, Stain Techn. *31*, 1 (1956).
[3] S. LAGERSTEDT, Exper. *12*, 425 (1956).
[4] N. JONSSON and S. LAGERSTEDT, Exper. *13*, 321 (1957).
[5] M. KUNITZ, J. biol. Chem. *164*, 563 (1946).

* Present address: Department of Zoology, Wilson College, Bombay (India).