Enzyme Histochemical Reactions of Mouse Ova

The biochemical events which occur at fertilization have indicated unique controlling mechanisms in the ovum. The increases in oxygen uptake at this time in the sea urchin egg[1,2] are correlated with a fall in ATP and a rise in ADP within the cytoplasm[3]. In the unfertilized sea urchin egg low levels of intermediates of the glycolytic pathways are present. This block in glycolysis is released 5 min after fertilization[4,5]. A possible mechanism of this type of control is spatial distribution of enzymes. For example, glucose-6-phosphate dehydrogenase is membrane-bound in the unfertilized egg, but appears in the soluble fraction of the cell after fertilization[6]. The following is an experiment in which histochemical localizations of dehydrogenases, ATPase (Ca^{++} activated), alkaline and acid phosphatases, cytochrome oxidase and NADH diaphorase were determined in mouse follicular and tubal ova.

Materials and methods. The dehydrogenases were detected by the method of NACHLAS et al.[7]. ATPase was tested for by the method of PADYKULA and HERRMAN[8]. KAPLOW'S[9] method was used for the localization of alkaline phosphatase and for acid phosphatase the technique of PEARSE[10] was used. Cytochrome oxidase was stained for using BURSTONE'S[11] technique.

Ovaries were removed from A-strain virgin mice aged 6 weeks and frozen on solid carbon dioxide. Sections 5 μ thick were cut in a Slee freezing microtome.

Spontaneously ovulated eggs from similar mice were obtained at metestrus: the Fallopian tube was dissected out, the segment containing ova was punctured and the eggs liberated directly into the incubation mixture of the histochemical assay. Superovulation was induced by the i.p. injection of pregnant mare's serum 10 IU (PMS) (Organon) followed by 5 IU human chorionic gonadotrophin (HCG) (Organon) 43 h later. The mice were killed 24 h after the second injection and eggs collected as before.

Homogenates of mouse ovaries and ova in Hanks' solution were applied to acrylamide gel for disc electrophoresis. Isoenzymes of lactate dehydrogenase were stained by a tetrazolium method[12].

Results and discussion. Staining for succinate dehydrogenase revealed little activity in the follicular ova, whilst the surrounding granulosa cells showed marked activity. In the tubal egg there was an eccentric area of enzyme activity which was not associated with the cell surface. The attached cumulus cells also showed succinate dehydrogenase activity, and this was spread throughout the cytoplasm. No isocitrate dehydrogenase (NAD^+) was present in the follicular egg, but was found in the tubal ovum. The cumulus cells reacted strongly with a well marked perinuclear band of particulate activity (Figure 1). Malate dehydrogenase was not detected in either preparation. Lactate and glutamate dehydrogenase and NADH diaphorase were present throughout the cytoplasm of follicular and tubal eggs. No α-glycerophosphate dehydrogenase and cytochrome oxidase were seen in follicular ova, but there was overall activity in the tubal eggs with large central clumps of granules. Similarly, glucose-6-phosphate dehydrogenase appeared to have a particulate activity in the tubal egg with an eccentric area of more intense activity. ATPase was present in both follicular and tubal eggs. The cumulus cells also showed a marked nuclear reaction. Acid phosphatase was abundant in follicular eggs but usually absent from tubal eggs. Alkaline phosphatase showed only slight activity in both types of ova.

The same electrophoretic pattern for lactate dehydrogenase in both ovarian homogenate and fertilized tubal eggs was seen. In both instances all 5 enzyme forms were present.

[1] O. WARBURG, Hoppe-Seyler's Z. physiol. Chem. *66*, 305 (1910).
[2] I. YASUMASU and E. NAKANO, Biol. Bull. *125*, 182 (1963).
[3] A. MONROY, *Chemistry and Physiology of Fertilization* (Holt, Rinehart & Winston 1965), p. 88.
[4] K. AKETA, R. BIANCHETTI, E. MARRÉ and A. MONROY, Biochim. biophys. Acta *86*, 211 (1964).
[5] A. MONROY and M. L. VITTORELLI, Experientia *16*, 56 (1966).
[6] N. ISONO, J. Fac. Sci. Tokyo Univ. Sect. 4, *10*, 37 (1963).
[7] M. M. NACHLAS, K. C. TSOU, E. DESOUZA, C. S. CHENG and A. M. SELIGMAN, J. Histochem. Cytochem. *5*, 420 (1957).
[8] H. A. PADYKULA and E. HERRMAN, J. Histochem. Cytochem *3*, 161 (1955a); *3*, 170 (1955b).
[9] L. S. KAPLOW, Blood *10*, 1023 (1955).
[10] A. G. E. PEARSE, *Histochemistry – Theoretical and Applied* (Churchill, London 1961), pp. 868, 874, 882 and 906.
[11] M. S. BURSTONE, J. Histochem. Cytochem. *9*, 59 (1961).
[12] J. H. WILKINSON, *Isoenzymes* (Spon, London 1965), p. 37.

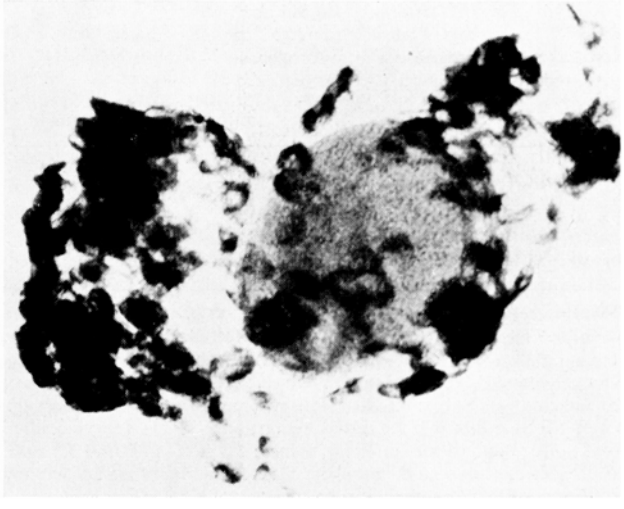

Fig. 1. Ovum and cumulus cells stained for isocitrate dehydrogenase. This is a tubal ovum which has been liberated from the Fallopian tube by puncture. × 350.

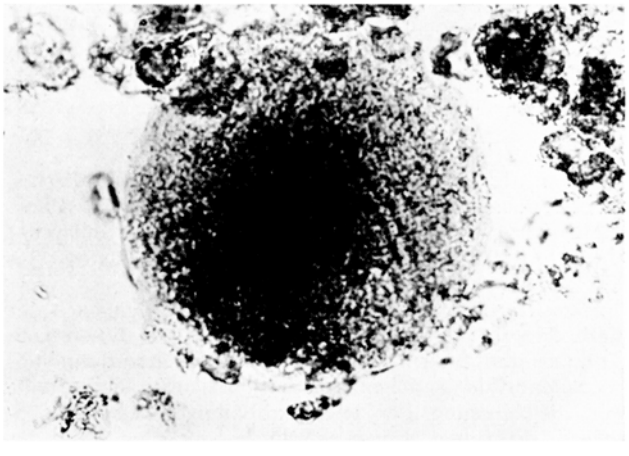

Fig. 2. Tubal ovum stained for cytochrome oxidase. The characteristic paracentral intense staining reaction is present. × 800.

The histochemical evidence indicates differences in amount and distribution of enzyme activity in the 2 types of ova. These could represent genuine alterations in biochemical regulatory mechanisms following ovulation. A structural basis for such changes in mitochondrial enzymes has been observed by ZAMBONI and MASTROIANNI[13], who showed that in early rabbit oocytes there are few, clustered mitochondria, whilst mature ova contain more numerous evenly distributed mitochondria. In the present work, however, an apparent increase in activity of lactate dehydrogenase was observed in superovulated eggs as compared with spontaneously ovulated eggs. This could be the result of gonadotrophin induced anomalies in the zona pellucida similar to those observed by KATZBERG and HENDRICKX[14] in the baboon and thought to enhance the permeability of the zona.

A structural explanation for the eccentric distribution of activity of some dehydrogenases within the tubal ovum would require an irregular distribution of mitochondria. This is seen in oocytes of human primordial follicles where mitochondria are concentrated in the crescent-shaped Balbiani's vitelline body[15]. In the penetrated tubal ovum when the pronucleus is forming, the organelles concentrate in the cytoplasmic area containing the chromosomes[16].

The concentration of ATPase activity at the periphery of the tubal oocyte is probably related to the role of the egg surface during cleavage. MIKI[17] suggests that the ATPase activity of the cortex of the sea urchin egg might be involved in the transformation of the chemical energy of ATP into the mechanical energy for cleavage. DALCQ[18] has shown that the furrows of cleaving mice eggs give a positive reaction when the eggs are incubated in the presence of nucleotides.

Zusammenfassung. Nachweis, dass in den Eiern im Mäuseeileiter grosse Quantitäten von ATPase, Cytochromaoxidase, Glukose-6-phosphat-dehydrogenase, Mitochondrialdehydrogenasen, saure und alkalische Phosphatasen lokalisiert sind. In den Eiern im Eierstock hingegen wurden ebenfalls ATPase, Phosphatase und Lactat-dehydrogenase festgestellt, obwohl von den Mitochondrialenzymen nur geringe Mengen gefunden wurden.

ANNETTE SKINNER and R. G. SPECTOR

Paediatric Research Unit and Department of Pharmacology, Guy's Hospital Medical School, London, S.E.1 (England), 25 April 1968.

[13] L. ZAMBONI and L. MASTROIANNI JR., J. Ultrastruct. Res. *14*, 95 (1966).
[14] A. A. KATZBERG and A. G. HENDRICKX, Science *151*, 1225 (1966).
[15] A. T. HERTIG and E. C. ADAMS, J. Cell Biol. *34*, 647 (1967).
[16] L. ZAMBONI and L. MASTROIANNI JR., J. Ultrastruct. Res. *14*, 118 (1966).
[17] T. MIKI, Expl Cell Res. *33*, 575 (1964).
[18] A. M. DALCQ, Archs Biol., Liège *73*, 405 (1962).

Über die Wirkung von Ethacrynsäure auf die Energieübertragung in Mitochondrien

Ethacrynsäure, ein saluretisch wirksames Phenoxyessigsäurederivat (2, 3-Dichlor-4-(2-methylenbutyryl)phenoxyessigsäure), hemmt die Na^+-, K^+- und Mg^{++}-abhängige sogenannte Membran-ATPase in vivo[1] und in vitro[2]. GAUDEMER und FOUCHER[3] konnten zeigen, dass die Substanz in Rattenlebermitochondrien eine energiegekoppelte Reaktion, nämlich die Reduktion von Acetacetat durch Sukzinat hemmt. Dieser Befund dürfte für den Wirkungsmechanismus von Ethacrynsäure von Bedeutung sein. Das Ziel der vorliegenden Arbeit war es daher, durch Untersuchung anderer energiegekoppelter mitochondrialer Reaktionen diese Wirkung von Ethacrynsäure weiter zu beleuchten, wozu uns die energieabhängige $NADP^+$-Reduktion durch NADH (energieabhängige Transhydrogenase-Reaktion) in submitochondrialen Teilchen geeignet erschien. Diese Reaktion

$$NADH + NADP^+ + \sim X \rightarrow NAD^+ + NADPH + X$$

benötigt als Energiequelle ein nicht phosphoryliertes, energiereiches Zwischenprodukt der oxydativen Phosphorylierung (symbolisiert durch $\sim X$), das entweder durch die Mitochondrienatmung oder durch zugesetztes ATP erzeugt wird[4].

Methodik. Rattenlebermitochondrien wurden nach der Methode von WEINBACH[5] gewonnen und aus diesen nach KIELLEY und BRONK[6] durch Ultraschallbehandlung submitochondriale Teilchen («sonic particles») hergestellt. Für die Messung der energieabhängigen Transhydrogenase-Reaktionen gingen wir im wesentlichen nach der Methode von DANIELSON und ERNSTER[4] vor, verwendeten aber statt Äthanol und Alkoholdehydrogenase als Wasserstoffdonatorsystem Laktat und Laktatdehydrogenase. Die Hemmung der Transhydrogenase-Reaktion durch Ethacrynsäure wurde sowohl unter anaeroben Bedingungen mit ATP als Energiequelle («ATP-abhängige Transhydrogenase») als auch unter aeroben Bedingungen

Hemmung energieabhängiger Transhydrogenase-Reaktionen durch Ethacrynsäure

Ethacrynsäure Konzentration (M)	(a) NADH-abhängige Transhydrogenase		(b) Sukzinat-abhängige Transhydrogenase		(c) ATP-abhängige Transhydrogenase	
	spez. Akt.	% Hemmung	spez. Akt.	% Hemmung	spez. Akt.	% Hemmung
–	12,6	–	14,1	–	22,1	–
5×10^{-5}	8,0	35	12,6	11	16,0	28
1×10^{-4}	4,5	64	11,6	18	15,1	32
1×10^{-3}	3,5	72	5,5	61	6,6	70
2×10^{-3}	1,0	92	2,3	84	1,8	92

Reaktionsmedium (Endvolumen: 3,0 ml). (a) NADH als Substrat: 50 mM *Tris*-Acetat-Puffer (pH 8,0), 6 mM $MgCl_2$, 250 mM Saccharose, 333 μM $NADP^+$, 33,3 μM NAD^+, 170 mM Laktat, 25 μg Kaninchenmuskel-Laktatdehydrogenase, 0,2 ml «sonic particles»; (b) Sukzinat als Substrat: Reaktionsmischung wie unter (a), ferner: 3 mM Na-Sukzinat und 3,4 μM Rotenon; (c) ATP als Energiequelle: Reaktionsmischung wie unter (a), ferner: 1,3 mM ATP und 3,3 mM KCN, 3,4 μM Rotenon. Messung der Extinktionszunahme bei 340 nm im Beckman DB-Spektralphotometer gegen eine Vergleichsküvette, die entweder kein $NADP^+$ (a), oder kein Sukzinat (b) oder kein ATP (c) enthält (Temperatur: 25 °C). Spezifische Aktivität ausgedrückt als gebildete mμMol NADPH/mg Protein/min.