CX. PROTEASES AND ONTOGENESIS.

I. CATHEPSIN IN THE CHICK EMBRYO.

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THE distribution and activity in the embryo of proteolytic enzymes, especially cathepsin and polypeptidases, have not hitherto attracted very much attention. In a paper on the activity of cathepsin in tumours, Krebs [1931] found only a small amount of cathepsin in chick embryos of 8 days' incubation, the proteolytic factor (PQ) being only 1.24, whilst in the most active tissue (rat kidney) he found PQ 25-30. Similar results have been obtained in a fragmentary way by Maschmann and Helmert [1933], Borger and Peters [1933] and others. The present paper describes a systematic investigation of cathepsin in the chick embryo.

EXPERIMENTAL.

The method of Krebs was applied with small modifications. The chick embryos, after being removed from the eggs, were washed several times with Ringer solution, then weighed and crushed in the mortar with a suitable amount of glycerol (100 ml. 87 % glycerol + 0.5 ml. glacial acetic acid), 2 ml. of glycerol being added to each gram of tissue. The mixture was left for 7 hours at room temperature and then for about 15 hours in the refrigerator at 0°. The extract so obtained was centrifuged and used as the enzyme extract. Gelatin purified by HCN and acetic acid was generally used as substrate, but in some experiments ovalbumin and lecithovitellin were used. To 5 ml. of 8 % gelatin, 1 ml. of enzyme extract was added, with either 1 ml. of 0.8 % cysteine or 1 ml. of water. At the beginning of the experiment and after incubation for 28-33 hours at 37°, the amino-N in 1 ml. of solution was determined by the Van Slyke method.

The activity of cathepsin is expressed as follows [Krebs, 1931]:

$$PQ = \frac{\mu l. N_2}{tv \frac{a}{5(a+b)}},$$

where t = time of action of enzyme, v = vol. of enzyme extract used in the experiment, a and b = vol. of tissue and of glycerol, respectively, used for extraction. Since in this equation the dry weight of tissue is calculated using factor 5 we had to change this in our calculations in order to get the true dry weight, which changes with age of the embryo [Needham, 1931].

In the first experiments the increase of amino-N in solutions of gelatin without enzyme and in corresponding enzyme extracts was also determined, but this increase was so small that it had no influence on the final results. Similar controls, however, were necessary in experiments with enzyme extracts from the yolk-sac.

Incubation of the hen's eggs was carried out in electrical incubators as described by Murray [1925], but the humidity was somewhat below optimum. The eggs were from pure-bred White Leghorn and Rhode Island Red hens.

RESULTS.

The optimum p_H for the cathepsin of the chick embryo agrees with that for cathepsin from other sources (Table I). Cysteine hydrochloride was used as enzymic activator. Oxidation of the cysteine was incomplete (nitroprusside

Table I. *Effect of p_H on activity of chick embryo cathepsin.*

Embryo of 7 days		Embryo of 9 days	
p_H	PQ	p_H	PQ
4.0	0.06	4.0	0.12
4.4	0.16	4.7	0.2
4.6	0.16	4.9	0.18
5.0	0.16		
5.2	0.16		
5.4	0.06		

reaction) even after 33 hours under the experimental conditions at p_H 4.7 and it was not necessary to make the experiments in absence of air. However, a few parallel experiments were performed under aerobic and anaerobic conditions and found the same PQ in both cases, *e.g.* 0.24.

Table II. *Change of activity of cathepsin during development.*

Days	PQ ₀	Average	PQ _{cyst}	Average
3½	0.26		0.37	
4	0.27		0.42	
	0.23	0.25	0.48	0.45
5	0.18		—	
	0.23		—	
	0.29	0.24	0.42	0.42
6	0.24	0.24	0.39	0.39
7	0.32		—	
	0.18	0.25	0.38	0.38
9	0.23	0.23	0.34	0.34
11	0.16		0.31	
	0.27	0.22	0.41	0.36
13	0.21	0.21	0.46	0.46
15	0.19	0.19	0.46	0.46
18	0.24		0.59	
	0.35		—	
18/19	0.22	0.27	0.28	0.43

During the whole life of the chick embryo the state of activity of cathepsin undergoes little change (Table II). Between 11 and 15 days' incubation the activity is perhaps a little smaller, but even these changes in PQ are hardly outside the experimental error. The catheptic activity is very small, whether activated by cysteine or not. In agreement with Maschmann we found a great part of the cathepsin in the activated state, this activation depending probably on the presence of —SH groups in the enzyme extract. We could always find a positive nitroprusside reaction in fresh enzyme extract, though sometimes it was very faint. It is interesting that the extent of activation by cysteine is nearly the same in all our experiments and very rarely more than 100 %. It seems to indicate that about half of the cathepsin in chick embryo tissues is in the activated state. But it is noteworthy that in our experiments the PQ is even lower than found by Krebs. On testing rat kidney we found a PQ of 16, as against 25–30 in Krebs's experiments. Probably these differences depend on the gelatin used.

Table III. *Activity of cathepsin in the yolk-sac.*

Days	PQ ₀	PQ _{cyst}
3½	2.48	5.34
4	2.57	4.74
5	3.3	6.1
12	3.2	5.8
18/19	2.8	6.8
18/19	3.5	7.2

It is very interesting that the activity of cathepsin in the yolk-sac (Table III) is much greater than in the embryo tissue. The average PQ of yolk-sac is about 3 and when activated by cysteine about 5–6. It seems probable, in view of the great synthetic task of embryonic tissues, that the work of decomposing the proteins takes place, not in the embryo itself, but in the surrounding membranes which send to the embryo the material necessary for building new tissues.

Experiments on the distribution of cathepsin in the embryo were performed by determining the activity of cathepsin in the head part and the tail part of the embryo separately. In both parts we were able to find cathepsin, as shown in Table IV.

Table IV. *Distribution of cathepsin in the embryo.*

	Embryo of 6 days		Embryo of 18 days	
	PQ ₀	PQ _{cyst}	PQ ₀	PQ _{cyst}
Total	0.14	0.29	—	—
Head part	0.07	0.19	0.23	0.24
Tail part	0.26	0.34	0.23	0.46
Liver	—	—	4.7	5.7
Kidney	—	—	2.2	4.2

In the embryo of 6 days the amount of cathepsin in the tail part is much greater than in the head part, but in the embryo of 18 days the PQ in both parts is the same. The apparent change in distribution probably depends on the development in the tail part of the embryo of heavy legs, feathers *etc.*, which do not contain cathepsin. In the liver and kidneys we found a comparatively greater amount of cathepsin, but not as great as in rat kidney.

Lecithovitellin (obtained from egg yolk by the procedure of Blackwood and Wishart [1934]) and ovalbumin were also used as substrates (Table V) as they

Table V. *Experiments with (a) lecithovitellin, (b) ovalbumin.*

Embryo of 9 days.			
	p _H	PQ ₀	PQ _{cyst}
(a)	4.0	0	0
	4.2	0	0
	4.5	0.12	—
	5.8	0.15	0.3
	6.7	0.1	0.18
(b)	4.7	0.24	0.36

seemed a more natural material for study of catheptic activity in the embryo. However, it was not possible to demonstrate any great decomposition of these egg proteins. With lecithovitellin the quotients obtained were even smaller than with gelatin, whilst the p_H optimum appeared to be shifted more to the alkaline side (to p_H 5.8). With ovalbumin in 2.4% solution, the PQ at p_H 4.7 was nearly the same as with gelatin, *e.g.* 0.24 (or 0.36 with cysteine) in the embryo of 7 days, 0.25 (or 0.36) in the embryo of 18 days.

Results indicating enzymic synthesis of protein under certain experimental conditions have been periodically reported. The importance of certain factors has been deduced: *e.g.* sufficient initial concentration of the products of decomposition [Wasteneys and Borsook, 1930], and some connection with oxidoreductive processes [Rondoni and Pozzi, 1933]. Besides this work on "plastein", work on the reversibility of cathepsin action has been described of recent years. It seemed that the embryonic tissue of the chick would be especially good material for such experiments. In such a series of experiments we used the following method. The gelatin (or ovalbumin) was digested by an enzymic extract from yolk-sac for 4-5 days at p_H 4.7, then concentrated and brought to p_H 6-7.2. Then the new enzyme extract from embryo was added. In some experiments we added H_2O_2 , glycogen or $CuSO_4$, substances inhibiting the decomposition of protein. At the beginning and at the end of the experiment amino-N and total protein (Kjeldahl) were determined. In certain cases it was possible to observe the disappearance of amino-N; but on observing all the conditions laid down by the above authors we could not find any synthesis of protein. Further experiments on this very interesting subject will be carried out.

DISCUSSION.

The problem of the utilisation of egg proteins by the embryo is not yet satisfactorily solved. It may be examined in two ways. First, we may examine the disappearance of proteins from white and yolk and their parallel increase in the embryo. Much work has been carried out along these lines, but we do not know accurately in what form the embryo obtains its material for building new tissues—whether in the form of polypeptide complexes or of simple amino-acids. From investigations on tissues growing *in vitro* we can only conclude that the mechanism of nitrogen assimilation is not very simple and it may or may not bear on the problem of the developing embryo.

The experiments of Carrel [1924] and especially of Willmer and Kendal [1932] show the importance of suitable material for this purpose. We have not merely to find the ways and products of decomposition of egg proteins, but also the form under which they are utilised by the embryo. The second line of attack is therefore to examine the enzymes of the whole embryonic system including the membranes. From our present experiments it may be concluded that the proteolytic activity of embryo tissues is very small. Apparently, polypeptidase activity (which, however, has not been examined throughout the whole period of embryonic life) is also not well developed in embryo tissues [Borger and Peters, 1933]. This suggests that decomposition of egg proteins takes place somewhere outside the embryo itself, probably in the yolk-sac. Unfortunately we cannot yet say how far this decomposition extends, or whether the products are "growth proteoses" or amino-acids.

Results showing the beneficial effect of small quantities of embryo extract in the presence of proteoses [Willmer and Kendal, 1932], and our own results seem to us to point to embryonic tissue as a suitable material for examining protein synthesis. But here the question arises as to the character of such synthesis; is it a synthesis of "ancestral" protein (Urprotein) not differentiated from simple amino-acids, as Alcock [1936] would suppose, or rather simply a reversal of cathepsin action as in plastein formation. The theoretical possibilities of such reversibility have been discussed by Borsook [1935], but the need for a source of energy for such synthesis still exists. We have tried to obtain a reversed action of cathepsin, but so far without success.

SUMMARY.

1. The activity of cathepsin in the chick embryo in connection with embryonic synthesis of protein was investigated.
2. The activity of cathepsin in the chick embryo (using gelatin, ovalbumin and lecithovitellin as substrates) is very small whether activated by cysteine or not. With lecithovitellin the p_H optimum is shifted more to the alkaline side.
3. The activity of cathepsin does not undergo much change during the whole developmental period of the chick embryo.
4. The yolk-sac is much more active (15–20 times) than the embryo.
5. No positive results on synthesis were obtained *in vitro*.

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