Studies on some aspects of the rôle of sulfhydryl groups in morphogenesis

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WITH ONE PLATE

The importance of sulfhydryl compounds in the process of morphogenesis was pointed out by Brachet (1950). Using specific inhibitors of -SH such as chloroacetophenone, it has been shown that embryos develop a number of malformations when the activity of -SH groups is interfered with (Beatty, 1951; Deuchar, 1957; Lakshmi, 1962a). It has been observed that the abnormalities are mainly in the nervous system and these findings are in agreement with the distribution of -SH groups in embryos of amphibia (Brachet, 1950) and chick (Rulon, 1935). These studies have vindicated the importance of -SH containing proteins stressed by Brachet. In the present report are described some studies using chloroacetophenone (CAP) as an -SH inhibitor and cysteine to reverse its action.

MATERIAL AND METHODS

Fresh fertilized hen's eggs were incubated to obtain the desired stage of development and explanted in vitro by the method described by New (1955). A stock solution of 0.01 M ω-chloroacetophenone (BDH) was prepared in 50 per cent. ethyl alcohol. This solution was freshly diluted with Pannett–Compton glucose saline (PC saline) to 5 · 10⁻⁴ M. For normal (untreated) controls 50 per cent. alcohol was similarly diluted for use in place of the CAP solution. Cysteine hydrochloride (BDH) was freshly dissolved in PC saline to prepare a 2.5 μg./ml. solution. The chemical solutions or plain PC saline were added on the ventral side of the blastoderm (ca. 0.2 ml. per embryo). Thin albumen was added around the ring. Embryos were grown in vitro for 20–22 hr., observed to record salient features and fixed in Bouin. Selected embryos were photographed as whole mounts after staining with haematoxylin. All the other embryos were sectioned serially and studied in further detail.

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Expt. 1. Reversal of the action of CAP

Embryos explanted at definitive primitive streak were treated with $5 \cdot 10^{-4}$ M CAP for 15 min. as described by Lakshmi (1962a). Following treatment with CAP the watch glass with the mounted embryo was plunged into ca. 300 ml. PC saline in a petri dish and the blastoderm was washed by jets of the solution with a medicine dropper. This precaution to remove all CAP outside the embryo was considered necessary because if any traces of it remained outside the embryo and reacted with cysteine added subsequently, it would give a false result of reversal. The CAP treated embryos were divided into two batches: (a) CAP controls, grown in PC saline added ventrally and thin albumen around the ring; (b) experimental embryos, grown in cysteine added ventrally and thin albumen around the ring. In all twenty-five CAP controls and twenty-five experimental embryos were studied. In addition to these, seventeen untreated controls (see above) and fifteen ‘master controls’ (cultured by the New method, without any treatment) were included in this experiment.

Expt. 2. Effect of CAP at head process stage

Embryos explanted at the fully formed head process stage were treated with CAP ($5 \cdot 10^{-4}$ M) for 15 min. and washed as described above. They were then grown with PC saline inside the ring and thin albumen around. In this experiment eighteen embryos were treated with CAP and ten embryos were kept as untreated controls.

Expt. 3. Reversal of CAP effects by a short treatment with cysteine

In this experiment embryos were treated with CAP alone (controls) and CAP followed by cysteine (experimental) as described under Expt. 1. However, the cysteine solution was replaced by PC saline at the full head process stage. In all twenty-three controls and twenty experimental embryos were studied in this experiment.

Expt. 4. Effects of delayed treatment with cysteine on CAP embryos

In this experiment embryos were treated with CAP as described in Expt. 1. After washing, control embryos were grown with PC saline inside the ring and thin albumen outside. Experimental embryos were also grown similarly till the full head process stage. The PC saline was then replaced by cysteine. This experiment included twenty-two controls and twenty-one experimental embryos.

RESULTS

When chick embryos are treated with CAP as described above, they develop a number of abnormalities, predominantly in the nervous system. Abnormalities produced by CAP in chick embryos have been described in detail by Lakshmi
Sulfhydryl groups in morphogenesis (1962a). When CAP treated embryos are subsequently treated with cysteine, they show normal development. A summary of the abnormalities caused by CAP and their alleviation by cysteine is given in the Table.

Embryos treated with CAP at the full head process stage develop quite normally. In embryos treated with CAP followed by cysteine, development is normal even if cysteine treatment is discontinued when the head process is fully formed (Plate, Figs. A–C). In sections also it is seen that development is normal in embryos treated with CAP followed by cysteine (Plate, Figs. E and F). When cysteine is applied at head process stage of embryos previously treated with CAP at definitive primitive streak, development is abnormal.

A few embryos were treated with cysteine alone (2–5 μg./ml. to) see if it has any effects on morphogenesis. It was found to be innocuous at this concentration.

**DISCUSSION**

Beatty (1951) found that abnormalities caused by CAP in amphibian embryos cannot be alleviated by a subsequent treatment with cysteine. When CAP and cysteine were used simultaneously, the effect depended on the resulting molarity of CAP calculated on the basis that the two chemicals react in equimolar

**Table**

Reversible effects of chloroacetophenone (CAP) on chick embryos cultured in vitro

<table>
<thead>
<tr>
<th>Organs showing malformations</th>
<th>Treatment: CAP</th>
<th>Treatment: CAP followed by cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fore brain</td>
<td>19 out of 25 (76%)</td>
<td>1 out of 25 (4%)</td>
</tr>
<tr>
<td>2. Posterior portion of brain</td>
<td>20 out of 25 (80%)</td>
<td>1 out of 25 (4%)</td>
</tr>
<tr>
<td>3. Neural tube, behind brain</td>
<td>14 out of 25 (56%)</td>
<td>None out of 25 (0%)</td>
</tr>
<tr>
<td>4. Optic vesicles (absence)</td>
<td>9 out of 25 (36%)</td>
<td>None out of 25 (0%)</td>
</tr>
<tr>
<td>5. Heart (absence)</td>
<td>10 out of 25 (40%)</td>
<td>None out of 25 (0%)</td>
</tr>
<tr>
<td>6. Somites (total absence)</td>
<td>4 out of 25 (16%)</td>
<td>None out of 25 (0%)</td>
</tr>
<tr>
<td>7. Subnormal length of embryonic axis</td>
<td>20 out of 25 (80%)</td>
<td>None out of 25 (0%)</td>
</tr>
</tbody>
</table>

**Explanation of Plate**

Fig. A. Embryo treated with chloroacetophenone (CAP) at definitive primitive streak.
Fig. B. Embryo treated with CAP at definitive primitive streak followed by a continuous treatment with cysteine.
Fig. C. Embryo treated with CAP at definitive primitive streak followed by treatment with cysteine up to head process only.
Fig. D. Embryo treated with CAP at definitive primitive streak followed by treatment with cysteine at head process stage.
Fig. E. Transverse section through an embryo treated with CAP at definitive primitive streak.
Fig. F. Transverse section of embryo treated with CAP at definitive primitive streak followed by treatment with cysteine.

Figs. A–D, × 16 (approx.); figs. E and F, × 225.
quantities and their product is harmless. From this Beatty (1951) concluded that CAP is an irreversible inhibitor of -SH groups. It is obvious from the results presented here that cysteine can reverse the various teratogenic effects of CAP in the chick embryo. The action of CAP is, therefore, not irreversible at least in the chick. Although it is not possible to explain why cysteine fails to reverse the effects of CAP on amphibian embryos, it may be suggested that the amphibian embryonic surface does not allow the two substances to penetrate with equal ease.

In considering the effects of -SH inhibitors on morphogenesis it is necessary to visualize the wide spectrum of the possible sites of their action. Sulfhydryl groups are important in the action of enzymes involved in oxidative metabolism. CAP is known to inhibit mitosis by acting as a spindle poison (Hughes, 1950). Evidence for the participation of some -SH containing protein in the transfer of activated amino acids from soluble RNA to ribosomes has been reported by von der Decken & Hultin (1960) and by Hülsmann & Lipmann (1960). Chamberlin & Berg (1962) have shown that intact -SH groups are necessary for the DNA-primed RNA synthesis in bacterial extracts. Disulfide (-SS-) bridges are essential to the secondary structure of many protein molecules and to their physiological properties. The oxido-reduction state of the -SH groups of proteins and its relation with free -SH containing substances like cysteine and glutathione in cellular metabolism are known to be important.

From the experimental results reported in the present work and also others reported earlier from our laboratory, one can eliminate some of the possible actions of CAP mentioned above. Lakshmi (1962a) has shown that CAP at 5 \times 10^{-4} \text{M} has no effect on the morphogenesis of chick embryos if administered for 15 min. at the head process stage. An identical treatment at the definitive primitive streak stage has profound effects on morphogenesis, especially on the developing neural tissue. Since these results were considered to be of some significance, we verified them in our Expt. 2. Without exception, all the embryos treated at the head process stage showed normal development. There seems to be no reason to consider that oxidative metabolism and synthesis of messenger RNA or proteins are not important after the head process has formed. In fact, these metabolic activities may be more important as the neural tissue begins to differentiate. The formation of a normal neural tube and its differentiation into neuromeres depends on a certain mitotic pattern in the various regions of the developing neural tube (Corliss & Robertson, 1963; Bergquist, 1964). It is possible that the CAP syndrome may arise from inhibition of mitotic activity. The possible action of CAP on mitosis is now being studied in some detail. A number of arrested mitoses are seen in the neural tube of the CAP syndrome. It is, however, surprising that there is no such effect on mitosis in case of embryos treated with CAP at the head process stage. The possibility of mitotic inhibition as a direct cause of abnormal development may be ruled out provisionally since treatment at head process stage has no effect on development. It is therefore
reasonable to assume that the effect of CAP on the embryos at the definitive primitive streak stage is not mainly on mitosis, enzymes of oxidative metabolism or synthesis of messenger RNA and proteins. The effect is probably on some system which is important for induction. From the results of our experiments 2, 3 and 4 it is clear that CAP affects some developmental process during the formation of head process. This supposition is borne out by the demonstration that Hensen's node considerably loses its inducing capacity as a result of treatment with CAP (Lakshmi, 1962b). Further, it has been shown (Lakshmi & Mulherkar, 1963) that the -SH content of Hensen's node decreases considerably after treatment with CAP.

Using colchicine, which also probably acts like CAP, similar results have been obtained in our laboratory (Diwan, 1964). It has been shown that colchicine is effective at definitive primitive streak stage while at head process stage it produces no malformations. He has also shown that the effects of colchicine can be reversed by a subsequent treatment with cysteine and that the inducing capacity of the node is affected by colchicine. Further, it has been demonstrated (Diwan & Mulherkar, unpublished) that the inducing capacity of colchicine treated nodes is restored by a subsequent treatment with cysteine.

Our results show that the -SH inhibitor acts in some way only during the stage definitive primitive streak to head process. From this it could be expected that alleviation of CAP effects should be possible even if cysteine treatment is discontinued after the head process has formed. On the other hand, cysteine should fail to ameliorate the CAP syndrome if added after the head process has formed. Experiments devised to test the validity of this reasoning have given the expected results (Expt. 3 and 4).

It is now generally held that the inducing substance is probably a protein. There is ample evidence that the inducing agent obtained from various adult tissue fractions is susceptible to proteolytic enzymes and is not affected by ribonuclease. Assuming that the inducing agent in the living embryo is also a protein we can visualize how CAP could affect the process of induction. CAP may act on the free -SH containing substances like cysteine or glutathione and change the oxidoreduction state in the intracellular milieu. This would result in alteration of the structure and properties of the protein molecules taking part in induction. Alternatively, CAP may act on the free -SH groups of these proteins and thus render them unable to give the inductive stimulus. Colchicine also probably acts in a similar manner. Further work is being undertaken to test the effects of -SH inhibitors on the process of induction.

**SUMMARY**

1. The effects of \(\omega\)-chlooroacetophenone (CAP), which is a powerful inhibitor of -SH, applied at \(5 \cdot 10^{-4}\) M for 15 min. at the definitive primitive streak and head process stages of chick embryos have been studied. It causes abnormalities
mainly in the developing neural tube when administered at definitive primitive streak stage while an identical treatment at the head process stage is innocuous.

2. The various effects of CAP can be completely reversed by a subsequent treatment with cysteine (2·5 µg./ml.) applied continuously or up to the head process stage only.

3. Cysteine fails to alleviate the effects of CAP if applied at the head process stage of embryos previously treated with CAP at the definitive primitive streak stage.

4. The possible modes of action of CAP have been discussed. It is suggested that CAP may alter the structure and physiological properties of protein molecules which take part in the process of embryonic induction.

RÉSUMÉ

Etude de quelques aspects du rôle des groupes sulfhydryles dans la morphogenèse

1. Cette étude porte sur les effets de l'ω-chloroacétophénone (CAP), puissant inhibiteur des liaisons -SH, appliqué à la concentration de 5·10⁻⁴ M pendant 15 min. sur l'embryon de Poulet au stade de la ligne primitive définitive et au stade du prolongement céphalique. Cette substance produit des anomalies spécialement sur le tube neural en développement quand elle est administrée au stade de la ligne primitive définitive tandis qu'un traitement identique au stade du prolongement céphalique est sans effet.

2. Les différents effets du CAP peuvent être complètement renversés par un traitement postérieur à la cystéine (2·5 µg./ml.) appliquée d'une manière continue ou seulement jusqu'au stade du prolongement céphalique.

3. La cystéine ne peut atténuer les effets du CAP si elle est appliquée au stade prolongement céphalique à des embryons traités au paravent au CAP quand ils étaient au stade ligne primitive définitive.

4. Les modes d'action possibles du CAP sont discutés. Il est suggéré que le CAP peut altérer la structure et les propriétés physiologiques des molécules protéiques qui prennent part aux processus d'induction embryonnaire.

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REFERENCES


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