The Effect of Chloroacetophenone on Chick Embryos Cultured *in vitro*

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INTRODUCTION

BRACHET's (1950) strong emphasis on the role of —SH-containing proteins in the process of induction has stimulated a study of the interference in the normal process of morphogenesis of chick embryos by chloroacetophenone, which has been described by Beatty (1951) as a specific and irreversible —SH inhibitor. He studied the effect of chloroacetophenone on the development of embryos of *Rana* and *Triturus* employing different concentrations. Deuchar (1957) also studied the action of the same chemical on the embryos of *Xenopus laevis* and has recorded abnormalities mainly in the brain and the eye. In the present work ω-chloroacetophenone (CAP) commercially known as phenacyl chloride (ω—$C_6H_5.CO.CH_2Cl$) was employed. The sample used was a B.D.H. product.

MATERIAL AND METHODS

Fresh fertilized hens' eggs brought from a local poultry farm were incubated at 37·5° C. for 16 to 18 hours to obtain definitive primitive-streak stages (range of length from 1·75 mm. to 2 mm.) or for about 22 hours to obtain head-process stages (average length of the head process alone 0·56 mm.).

The glass-ware used in the culturing of embryos was sterilized. The solutions employed for Pannett–Compton saline were separately autoclaved.

The embryos were explanted from the incubated eggs by the method of New (1955). Since chloroacetophenone is insoluble in water at room temperature a 0·02 M stock solution was made in 50 per cent. ethyl alcohol. The stock solution employed for controls was 50 per cent. ethyl alcohol without the chemical. In actual experiments the experimental stock solution and the control stock solution were diluted with fresh Pannett–Compton saline to the desired strength. In the experimental embryos diluted CAP solution (0·1 ml.) was placed on to the explanted embryos. Similarly, the diluted control solution (0·1 ml.) was placed on to the control embryos. The treatment was continued for various durations. Concentrations of 0·003 M, 0·002 M, 0·0016 M, 0·00125 M, 0·001 M, and 0·0005 M CAP were each tested for 120, 60, 30, and 15 minutes. After treatment the embryos were washed carefully to remove all the traces of CAP, and 0·1 ml.

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of fresh Pannett–Compton solution was added inside the ring. This albumen was placed surrounding the ring for nutrition. The embryos were incubated further for 21 hours. The above-mentioned experiments, designed to determine the highest concentration of CAP in which the embryos could differentiate, were done on 144 experimental embryos and 80 embryos were kept as controls. Since the solution used for the controls contained alcohol, for the batch of experimentals for 0·003 M (CAP) a second set of controls was kept in which 0·1 ml. of fresh Pannett–Compton solution only was added. Albumen was added as usual for nutrition. This second set of controls was kept in order to ensure that the controls treated with alcohol lived normally. In fact, it was observed that there was no difference whatever in the development of embryos of the two sets of controls. It will be helpful to note in this context that 0·1 ml. of the control solution of final concentration corresponding to 0·003 M CAP contains 0·005 ml. of 100 per cent. alcohol and 0·1 ml. of the final dilution of the control solution corresponding to 0·0005 M CAP (the final and lowest concentration) contains as little as 0·00125 ml. of 100 per cent. alcohol. The alcohol present in the control solution is therefore negligible. It was observed, by the above procedure, that only the treatment with 0·0005 M CAP for 15 minutes was suitable and allowed the embryos to differentiate. Therefore this treatment was tried on 91 embryos at the primitive-streak stage and 13 at the head-process stage. Sixty-seven embryos at the primitive-streak stage and 13 at the head-process stage served as controls. Following 21 hours' incubation of the treated embryos, they were thoroughly examined, lengths were measured, malformations recorded, and they were then fixed in acetic-alcohol for $1\frac{1}{2}$ hours. Some were processed for whole mounts, stained in dilute Delafield's haematoxylin, and photographed. They were later sectioned serially at 10 μ, and examined histologically.

RESULTS

Out of 91 primitive-streak embryos 21 died and 70 survived. The analysis of the abnormalities shown by them is given in Table 1 and that of the controls is given in Table 2.

The morphological descriptions of the entire embryos were noted and the lengths of the control and the treated embryos were measured before fixation. These measurements were later confirmed in the whole mount preparations. From the observations made before fixation of the embryos, from stained whole mounts, and from sections, the final descriptions were written. The descriptions of the speciments illustrated are given below.

TEXT-FIG. 1

In this specimen the brain has not differentiated. The anterior-most region has formed a short uniform cylindrical mass of neural tissue. In this mass a cavity gradually appears which is absent in the anterior-most region (Text-fig. 2). The region corresponding to the forebrain (level B) shows a narrow, slit-like

TABLE 1

Analysis of abnormalities produced by CAP in chick embryos at the primitive-streak stage

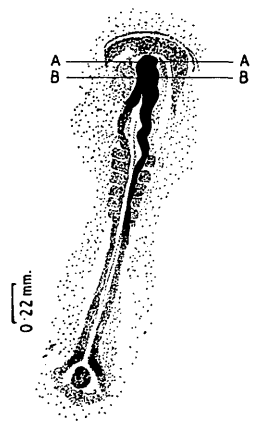
		No. of embryos	*Percentage*		*No. of embryos*	*Percentage*
Forebrain	Abnormal	56	80·0	Normal	14	20·0
Posterior portion of brain	Abnormal	57	81·4	Normal	13	18·6
Neural tube	Abnormal	34	48·7	Normal	36	57·3
Optic vesicle:						
(*a*) Both absent		31	44·2			
(*b*) Both present	Abnormal	19	28·4	Normal	14	20·0
(*c*) Only one present	Right	3	3·7	Left	3	3·7
Heart:						
(*a*) Vitelline veins not fused		16	22·8			
(*b*) Vitelline veins fused. Flexure	Absent	27	38·5	Present	27	38·7
Notochord	Abnormal	14	20·0	Normal	56	80·0
Somites:						
(*a*) Formed	Abnormal	11	15·7	Normal	43	61·5
(*b*) Not formed		16	22·8			
Length of axis	Short	62	88·5	Nearly normal	8	11·5

TABLE 2

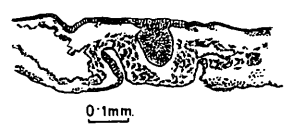
Analysis of abnormalities in control embryos

		No. of embryos	*Percentage*		*No. of embryos*	*Percentage*
Forebrain	Abnormal	Nil	Nil	Normal	67	100
Posterior portion of brain	Abnormal	Nil	Nil	Normal	67	100
Neural tube	Abnormal	Nil	Nil	Normal	67	100
Optic vesicle:						
(*a*) Both absent		8	11·9			
(*b*) Both present	Abnormal	Nil	Nil	Normal	59	88·1
(*c*) Only one present		Nil				
Heart:						
(*a*) Vitelline veins not fused		16	23·9			
(*b*) Vitelline veins fused. Flexure	Absent	Nil	Nil	Present	51	76·1
Notochord	Abnormal	Nil	Nil	Normal	67	100
Somites:						
(*a*) Formed	Abnormal	Nil	Nil	Normal	67	100
(*b*) Not formed		Nil				
Length of axis (average 5·5 mm.)	Below average	20	29·85	Average and above	47	70·15

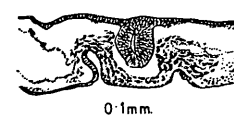
lumen (Text-fig. 3). The notochord has started from the middle of the cylindrical mass probably indicating the level of the midbrain. The notch discernible on the right side of the cylindrical portion probably marks the posterior boundary of the forebrain. The neural folds are wavy and wide apart. In the region of the somites the neural folds have come closer though they have not united. There are 6 pairs of somites which are almost normal. The heart is not formed and shortening of the axis is seen. The length of the embryo is 3 mm. and that of the corresponding control is 5 mm.



TEXT-FIG. 1. Macroscopic view of chick embryo treated with 0·0005 M CAP for 15 minutes at the primitive-streak stage.



TEXT-FIG. 2. Transverse section through the anterior cylindrical mass representing the brain, passing approximately through level *A*.



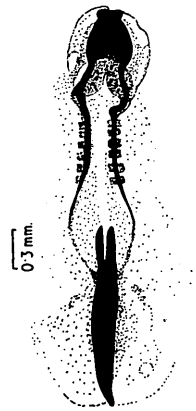
TEXT-FIG. 3. Transverse section through the anterior cylindrical mass representing the brain, passing approximately through level *B*.

TEXT-FIG. 4

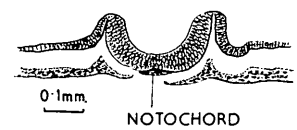
This specimen shows a closed brain region which does not show the three main divisions, though a slight division into two is seen. The lumen of the brain is normal. The neural tube is wide and open. Fore-gut is formed. In the anterior region the notochord is slightly hypertrophied, the size gradually reduces and it becomes flattened (Text-fig. 5). Six pairs of somites are seen. The length of the embryo is 4 mm., the corresponding control length is 5 mm.

TEXT-FIG. 6

The specimen shows a widely opened brain portion with two divisions. The constriction between these two regions is very well marked. The anterior portion gives rise to a small protuberance on the left side which may be the left optic vesicle (Text-fig. 7, section reversed). The second division may represent both the mid- and hindbrain regions. The neural plate in this region is almost flattened (Text-fig. 8, section reversed). Continuous posteriorly is a wavy irregular neural

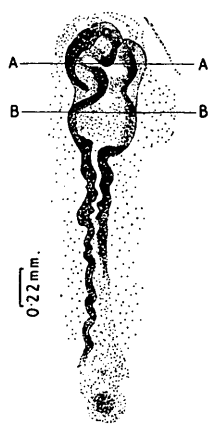


TEXT-FIG. 4. Macroscopical view of chick embryo treated with 0·0005 M CAP for 15 minutes at the primitive-streak stage.

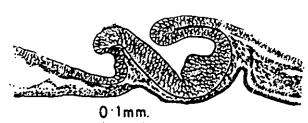


TEXT-FIG. 5. T.S. of embryo of Text-fig. 4 showing flattening of the notochord and wide open neural tube.

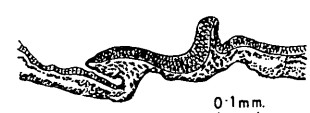
tube which is open. No somites are formed, and the fore-gut is not seen. The length of the axis is 3 mm., the corresponding control measures 5 mm.



TEXT-FIG. 6. Macroscopic view of chick embryo treated with 0·0005 M CAP for 15 minutes at the primitive-streak stage.



TEXT-FIG. 7. T.S. passing through level *A* of embryo in Text-fig. 6 showing probably the left optic vesicle (section reversed).



TEXT-FIG. 8. T.S. passing through level *B* of embryo in text-fig. 6 showing flattening of neural plate (section reversed).

TEXT-FIG. 9

The brain of this embryo is very abnormal. The anterior-most region, which is constricted off from the immediately posterior region, is wide open. This is probably the forebrain. A tendency towards the formation of optic vesicles is observed in sections. The region immediately following is mildly grooved into two portions. Only this region of the brain is closed. It is also shifted to the left.

The lumen in this region looks normal. The neural folds are wide apart and wavy. Fore-gut is formed. Three pairs of indistinct somites are seen. The length of the embryo is 2·75 mm., and the length of the corresponding control is 4 mm. Sections, unfortunately, could not be photographed.

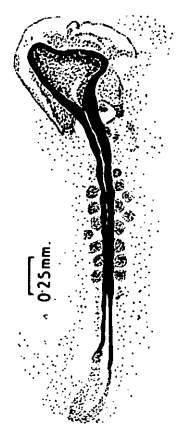
TEXT-FIG. 9. Macroscopic view of chick embryo treated with 0·0005 M CAP for 15 minutes at the primitive-streak stage.

TEXT-FIG. 10

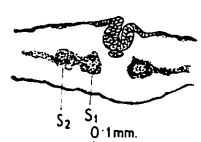
The brain in this case is not normal, a perfect division into the various parts is not observed. The optic vesicles are formed but are distorted in position. The lumen of the brain is normal. The neural tube is also normal. Splitting of somites is seen resulting in the formation of supernumerary somites (Text-fig. 11, section reversed). On the right side of the third right somite two extra somites are seen. On the left in between the 4th and 5th there is an extra somite. This is the only case which showed the somite abnormality of the type described. Fore-gut is normal. Vitelline veins are fused to form the endothelium of the heart. The length of the embryo is 3 mm., the corresponding control measures 6 mm.

For comparison with the entire mounts one control embryo is illustrated (Text-fig. 12).

It has been observed that the embryos at the head-process stage treated with CAP showed normal development.



TEXT-FIG. 10. Macroscopic view of chick embryo treated with 0·0005 M CAP for 15 minutes at the primitive-streak stage. Presence of supernumerary somites to be noted.



TEXT-FIG. 11. T.S. passing through the 3rd somite level of embryo in Text-fig. 10. Splitting of somites (S_1 and S_2) on the right side to be noted (section reversed).

TEXT-FIG. 12. Control chick embryo for experiments of treatment of chick embryos at the primitive-streak stage with 0·0005 M CAP for 15 minutes.

DISCUSSION

The effects on morphogenesis of chemicals whose action and properties are known could be interpreted as an indication of the presence or distribution of the probable target substances. Thus the application of chemical substances has become a means of studying morphogenesis.

A reference to Table 1 shows that the brain region was abnormal in 81·4 per cent. of our cases. The posterior portion of the brain is abnormal in 81·4 per cent. and forebrain in 80 per cent. of the cases. Attention is drawn to these percentages of abnormality in view of the opinion held that the forebrain differentiates autonomously (Waddington, 1932; Rudnick, 1938; Spratt, 1942, 1947). The lack of differentiation of the brain into the prosencephalon, mesencephalon, and the rhombencephalon was a common occurrence. Text-fig. 1 represents a case where the suppression of all the three parts of the brain seems to have taken place. Many cases occurred in which the brain was found to be open throughout (Text-fig. 6), or open only in the forebrain region (Text-fig. 9). Deuchar (1957) studied the effect of chloroacetophenone at the concentrations of 16 and 20×10^{-6} M on early embryos of *X. laevis*. She observed that embryos treated at early neurula stages later showed absence of parts of the brain. In some cases the whole brain was absent whereas in others only the hindbrain was absent. She has also mentioned a specific case in which the embryo lacked the forebrain. Defects in the central nervous system were also produced by Stockard (1910), Werber (1916), Töndury (1937), Hall (1942), and others using different chemicals.

In Text-fig. 1 probably the open region corresponds to the posterior portion of the hindbrain. Reference has already been made to complete or partial closure of the brain region observed in the experimental embryos. The chemical seems to have affected the movements leading to the closure of the brain. Brachet (1958) and Brachet & Delange-Cornil (1959) have mentioned similar inhibition of closure of neural plate as an effect of β-mercapto-ethanol on amphibian eggs. Further, Brachet (1960), reviewing the work done with —SH inhibitors, said that 'they produce profound alterations in the nervous system, which does not close properly and which remains abnormally thick'.

Deuchar (1957) observed that CAP caused numerous eye defects like reduction or absence of eyes and also structural defects. In the present work there have been cases in which only the left (Text-figs. 6 and 7) or the right optic vesicles were present.

Abnormalities of the neural tube consisted mainly in its open nature and its curved or wavy path. A few notochordal abnormalities were found (20 per cent.) which consisted chiefly in dorso-ventral flattening (Text-fig. 5), hypertrophy, and shifting ventralwards in some cases.

Shortening of the axis was a common feature in the treated embryos. In fact not a single embryo was normal in length as compared to the controls. Cases

mentioned as nearly normal in Table 1 fell within 0·5 mm. of the length of the control.

It has already been mentioned that CAP at 0·0005 M had no effect on embryos at the head-process stage. Although this was observed only in 13 embryos it is not a matter of chance that all the 13 should show normal development. In these embryos the invagination of the chorda mesoderm has already taken place and the process of induction of nervous system has also taken place so that synthesis of specific proteins of neural tissue has been initiated and is being continued. This might be the reason why the head-process stage embryos did not show any abnormalities.

The abnormalities in the nervous system especially in the brain region are consistent with the distribution of —SH groups studied by Brachet (1938) in Amphibia. Rapkine & Brachet (1951) obtained inhibition of formation of nervous system in dorsal explants using oxidized glutathione or alloxane to oxidise the —SH groups.

It is relevant to mention here the studies of Feldman & Waddington (1955) on the incorporation of methionine S^{35} in chick embryos. They found maximum incorporation of the amino-acid in the brain region which is also the region claimed by Brachet to contain abundant —SH-containing proteins. Further, the brain region had more labelled amino-acid incorporated than the posterior. This is of interest in view of the indication given by the authors that methionine is the precursor of other sulphur-containing amino-acids. The results obtained with CAP treatment, namely malformations of brain in 81·4 per cent. of the cases and less in the neural tube (48·7 per cent.), seem to be consistent with the observations of Feldman & Waddington (1955) and of Brachet (1938, 1950).

SUMMARY

1. The effect of chloroacetophenone (0·0005 M), which has been described as a powerful and irreversible —SH reactant, has been studied on chick embryos cultured *in vitro* at the primitive-streak and head-process stages.

2. Abnormalities were produced predominantly in the brain region, and included improper differentiation, partial or complete non-closure, and defective lumen. Neural tubes were irregular and open. Shortening of the axis was also common.

3. Embryos at the head-process stage were not affected by the substance at the concentration used.

4. The effect of the chemical on the morphogenesis of the chick embryo is discussed.

RÉSUMÉ

Effet de la chloroacétophénone sur l'embryon de Poulet cultivé in vitro

1. L'effet de la chloroacétophénone (0·0005 M), qui passe pour réagir fortement et de façon irréversible avec les groupements —SH, a été étudié sur des

embryons de Poulet cultivés *in vitro* au stade de la ligne primitive et du processus céphalique.

2. Des anomalies sont obtenues principalement des régions cérébrales pouvant présenter une différenciation anormale, une non-fermeture complète ou partielle ou une absence de canal. Le tube neural est irrégulier, et reste ouvert. Le raccourcissement de l'axe est fréquent.

3. Les embryons au stade du processus céphalique n'ont pas manifesté d'effet du traitement, à la concentration utilisée.

4. L'effet de la substance utilisée sur la morphogenèse de l'embryon de Poulet est discutée.

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