THE ANTAGONISM BETWEEN ACETIC ACID AND THE CHLORIDES OF SODIUM, POTASSIUM, AND CALCIUM AS MANIFESTED IN DEVELOPING FUNDULUS EMBRYOS.

BY PHILIP B. ARMSTRONG.

(From the Marine Biological Laboratory, Woods Hole, and the Department of Anatomy, Cornell University Medical College, New York.)

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Loeb in his experiments (1, 2, 3) on acid-salt antagonism found that the embryos of developing *Fundulus* eggs immersed in acetic acid (M/500) were soon killed, and that the addition of certain amounts of any one of several salts served to protect the embryos from the toxic action of the acid. Loeb considered that the antagonism of the acid by the salt took place at the chorionic membrane; that the presence of the salt prevented or slowed the penetration of the acid through this membrane. He cites no control experiments on embryos of the same age from which the chorionic membranes were removed. In this paper are described the results of such control experiments together with one additional experiment. They materially modify the interpretation of Loeb's original experiments and especially his ideas as to the site and mechanism of the antagonism and its relation to permeability.

Material and Methods.

Embryos of *Fundulus heteroclitus* 5 to 8 days old were used in the experiments. By this stage the blastopore has closed and none of the body orifices have broken through so the entire external surface of the embryo is a continuous intact layer of ectoderm. The chorionic membrane which surrounds the embryo was removed under a binocular dissecting microscope with dissecting needles and iridectomy scissors. In removing the membrane, special precautions must be taken to avoid injury to the embryo. The following procedure gave uniformly good results: The point of a sharp dissecting needle was pushed into the membrane and the egg rotated so that the tip of the needle within the membrane could be held against the bottom of the dish at an acute angle. A second needle was then drawn across the under side of the first needle so as to make a slit in the membrane large enough for the introduction of the point of the lower blade of the iridectomy scissors. By this means the membrane was readily removed, without exerting any pressure on the embryo. The naked embryos were kept overnight in sea water, during which time a few embryos which had been injured in the removal of the membrane died. The mortality was usually 4 to 5 per cent.

In the experiments recorded in this paper M/500 acetic acid was used in combination with varied concentrations of Na, K or Ca chloride. The mixture was made by adding 1 cc. of M/10 acetic acid to 50 cc. of the salt solution which gave a M/500 concentration of acetic acid without changing appreciably the concentration of the salt solution. Into each acid-salt mixture ten naked embryos were placed after having been previously rapidly transferred through two changes of distilled water to avoid carrying over an appreciable quantity of sea water. For controls other embryos were placed in distilled water and in M/500 acetic acid.

EXPERIMENTAL.

1. Standstill of the Heart in Relation to Other Manifestations of the Toxic Action of Acetic Acid.

Loeb's criterion of the toxic action of the acid was standstill of the heart. In a preliminary experiment we will consider the standstill of the heart in relation to other manifestations of the toxic action of the acid.

If naked embryos are immersed in M/500 acetic acid, no change from the normal is noted during about the first half hour. They remain motionless except for an occasional twitch of the tail. After the first half hour some of the embryos show evidence of irritation by more frequent and vigorous tail movements. Soon after this the tips of the tails of these embryos become white and opaque. This whitening and opacity gradually spreads until at the end of 3 hours the entire tail is involved. The heart remains normal until after about half of the tail has become white and opaque. The pericardium then starts to shrink and the heart is slowly retracted until it is almost all under the head. During this procedure the rhythm, conduction and contractility of the heart become severely and variously affected. Fig. 1 shows the time relation between the onset of tail injury and standstill of the heart. The primary manifestation of the toxic action of the acid therefore appears to be the injury to the tail of the embryo and this may be used as a criterion of the injurious action of the acid. Stoppage of the heart beat is always secondary to the injury of the ectoderm of the tail. Embryos in acid show no disturbance of heart action so long as the surface of the embryo remains uninjured. Another constant feature of standstill of the heart as induced by acid is shrinkage of the pericardial cavity and retraction of the heart under the head. In addition, the standstill is complete and irreversible. It will be seen in a later experiment that the characteristics of heart stoppage of embryos immersed in KCl are very different from those in acid.

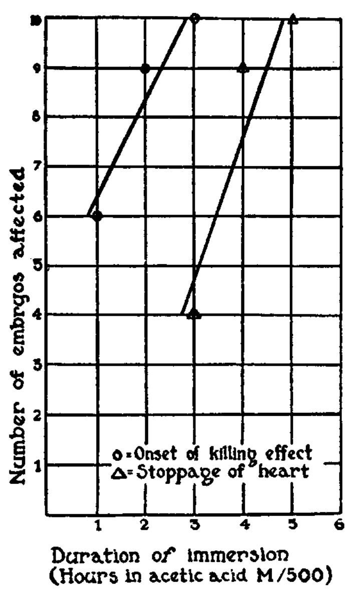

FIG. 1. Time relation between the onset of injury of the embryo and the stoppage of the heart in the acetic acid M/500.

2. The Antagonism of Different Concentrations of NaCl to the Action of Acetic Acid.

In this experiment naked embryos were immersed in NaCl-acetic acid mixtures. 1 cc. of M/10 acetic acid was added to 50 cc. of each different concentration of NaCl. This dilution gives M/500 acetic acid and the concentration of the salt solution is not appreciably changed. Table I shows that the higher concentration of the salt protected the naked embryos from the toxic action of the acid.

The results of this experiment are essentially the same as those reported by Loeb from his experiments with developing embryos enclosed within intact egg membranes. Apparently, therefore, the egg membrane plays no appreciable rôle in the antagonistic mechanism. In this experiment as in the preceding one, heart stoppage was always preceded by surface injury of the embryo.

TABLE I.

Antagonism of a Constant Concentration of Acetic Acid by Different Concentrations of Sodium Chloride.

Hrs. in solution	No. of embryos in which the heart continued to beat when immersed in 1 cc. of M/10 acetic acid in 50 cc. of:						Controls in distilled water
	Distilled water	M/64 so NaCl	M/32 sol NaCl	M/16 sol NaCl	M/8 sol NaCl	M/4 sol NaCl	
3	10	10	10	10	10	10	10
4	8	2	8	10	10	10	10
5	3	0	0	10	10	10	10
6	0			10	10	10	10
7				9	10	10	10
8				4	10	10	10
9				1	10	10	10
11				0	10	10	10
19					8	10	10
26					7	10	10
44					4	7	10

3. The Antagonism of Different Concentrations of $CaCl_2$ to the Action of Acetic Acid.

According to Loeb different salts in the same concentration were not equally effective in antagonizing the toxic action of acetic acid, *e.g.*, he found $CaCl_2$ to be much more effective than NaCl. Table II shows the effectiveness of $CaCl_2$ in protecting naked embryos from the toxic action of acetic acid.

It can be seen that the higher concentrations of $CaCl_2$ behave like those of NaCl but that a M/16 solution of $CaCl_2$ is more effective than a M/4 solution of NaCl. Since this is the same effect reported by Loeb for developing embryos enclosed within intact membranes, the difference in the effectiveness of these salts cannot be due to the presence of the membrane.

4. *The Difference in the Way the Heart Stops in KCl and in Acetic Acid.*

If developing *Fundulus* eggs are immersed in either a solution of KCl or acetic acid, the heart stops beating. However, the mode of stoppage for each is specific. In a normal 5 to 8 day *Fundulus* embryo the heart shows a regular conduction of all beats from the sinus to the auricle and thence to the ventricle. If the embryo is placed in a KCl solution, this regularity is soon upset, so that only every other beat is carried through to the auricle and ventricle, and the ratio of beats in sinus-auricle-ventricle becomes 4–2–2. With continued immersion more of the beats in the auricle and ventricle are suppressed

TABLE II.

Antagonism of a Constant Concentration of Acetic Acid by Different Concentrations of Calcium Chloride.

Hrs. in solution	No. of embryos in which the heart continued to beat when immersed in 1 cc. M/10 acetic acid in 50 cc. of:					Controls in distilled water
	Distilled water	M/1024 $CaCl_2$	M/256 $CaCl_2$	M/64 $CaCl_2$	M/16 $CaCl_2$	
2	10	10	10	10	10	10
3	6	8	10	10	10	10
4	0	4	10	10	10	10
6		1	8	10	10	10
9		0	6	10	10	10
20			2	3	10	10
72			0	0	10	10

and the ratio becomes 4–2–1 or possibly 8–4–1. Finally the auricle and ventricle stop beating and the beating then persists only in the sinus. Nor does the entire sinus contract for the pulsation becomes restricted to a small bit of tissue about midway between the venous and auricular ends of the sinus. In the KCl solution there is no evidence of any surface injury to the embryo nor is there any shrinkage of the pericardial cavity and, if the embryo is returned to sea water, normal heart function becomes reestablished. In brief, stoppage of the heart by KCl may be identified by the manner in which it takes place, by absence of surface injury and pericardial shrinkage, by persistence of pulsation in sinus and by reestablishment of normal heart function on return of embryo to sea water. On the other hand the action of acetic acid on the heart is preceded by surface injury of the embryo, the stoppage proceeds in no well defined steps, is accompanied by pericardial shrinkage, is complete and there is no reestablishment of the heart beat on returning the embryo to sea water.

5. The Antagonism of Different Concentrations of KCl to the Action of Acetic Acid.

Table III illustrates the antagonism of KCl to the surface-killing effect of the acid. The criterion of acid action is injury of the tail of the embryo as indicated by whitening and opacity. By the end of 3

TABLE III.

Antagonism of the Killing Effect of a Constant Concentration of Acetic Acid by Different Concentrations of Potassium Chloride.

Hrs. in solution	Embryos showing onset of injury in 1 cc. M/10 acetic acid in 50 cc. of:						Controls in distilled water
	Distilled water	M/64 KCl	M/32 KCl	M/16 KCl	M/8 KCl	M/4 KCl	
1	0	0	0	0	0	0	0
2	1	5	4	0	0	0	0
3	10	10	9	0	0	0	0
4			10	3	0	0	0
5-½				9	0	0	0
10				10	3	0	0

hours the hearts had stopped in all the solutions. In acetic acid plus M/64 KCl the mode of stoppage was characteristic of the acid effect, *viz.*, it was preceded by surface injury and was accompanied by shrinkage of the pericardial cavity, and was complete. In acetic acid plus M/4 KCl the mode of stoppage was characteristic of the KCl effect, *viz.*, the surface of the embryo remained uninjured, the stoppage was reached through definite steps, the pulsation persisted in the sinus and the pericardial cavity did not shrink. From this it follows that antagonism does not necessarily involve non-penetration of the salt for the M/4 KCl antagonized the surface killing effect of the acid but at the same time it penetrated and stopped the heart.

DISCUSSION.

Loeb (1, 2, 3) believed that several neutral salts including NaCl and $CaCl_2$ act similarly in that each can retard the rate of diffusion of acetic acid through the membrane of the developing *Fundulus* egg and so prevent or retard the toxic action of the acid on the embryo. If this is true then removal of the egg membrane should remove the protective action of the salt. In the three series of experiments described in this paper it was found that the protective action of the salt was as effective as ever after removal of the egg membrane. The effectiveness of this protection with NaCl was of the same order as that found by Loeb with the intact developing egg. With both intact eggs and naked embryos $CaCl_2$ was more strongly protective than NaCl. It is therefore unnecessary to attribute to the egg membrane any rôle in acid-salt antagonism since the results are quantitatively and qualitatively the same on naked embryos as on intact developing eggs of the same age. In a previous paper (5) we showed that, if intact eggs were immersed in either sea water or distilled water, the pH in the subchorionic space became that of the surrounding medium. The pH in the pericardial cavity, however, remained constant. It was suggested that it was not at the chorionic membrane that acid-salt antagonism took place. During the past season, Bodine has studied the action of Na, K and Ca chlorides on intact developing eggs and on embryos freed of their egg membranes. He states in a preliminary report (6) that the explanation of salt antagonism as advanced by Loeb needs certain corrections. In his experiments Loeb used the newly hatched fish as controls and found them to be very sensitive to acid-salt mixtures. However, such control material cannot be compared with the younger embryos. In the newly hatched fish the mouth and gills have broken through and the delicate surfaces of the gills are exposed to the action of the acid-salt mixtures whereas in the younger embryos the surface is still a continuous intact layer of ectoderm.

We must conclude, therefore, that the surface of the embryo is presumably the site of the acid-salt antagonism. It has been shown in this paper that the primary effect of the acetic acid was to injure the surface of the embryo. Stoppage of the heart was through secondary penetration of the acid. The presence of the salt prevented heart stoppage by preventing the surface injury. The antagonism at the surface of the embryo is not one in which the acid and salt each prevents the penetration of the other, which was Loeb's conception of the mechanism at the egg membrane. In the experiments in this paper on acetic acid-KCl antagonism, the higher concentrations of KCl antagonized the action of the acid on the surface of the embryo but at the same time the KCl penetrated and stopped the heart in the manner which is characteristic of KCl. That this stoppage was probably due to the penetration of KCl is further indicated by the fact that it is like that of the isolated terrapin heart immersed in KCl (4).

SUMMARY.

1. Developing *Fundulus* embryos react in much the same way to mixtures of acetic acid in salt solutions whether their membranes are removed or not. It is therefore not necessary to assume any specific rôle for the membrane.

2. The primary toxic effect of acetic acid is to kill the surface of the embryo; heart stoppage is due to penetration of the acid after the surface has been injured.

3. The salt (NaCl, KCl, $CaCl_2$) antagonizes the acetic acid by slowing or preventing the killing of the surface of the embryo.

4. If embryos are immersed in certain KCl-acetic acid mixtures, the surface killing effect of the acid is antagonized but at the same time the KCl penetrates and stops the heart in the manner which is characteristic of a KCl solution alone.

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BIBLIOGRAPHY.

1. Loeb, J., On the rôle of electrolytes in the diffusion of acid into the egg of *Fundulus*, *J. Biol. Chem.*, 1915, xxiii, 139.
2. Loeb, J., The diffusion of electrolytes through the membranes of living cells. V. The additive effect of salt and base and the antagonistic effect of salt and acid, *J. Biol. Chem.*, 1917, xxxii, 147.

3. Loeb, J., Sodium chloride and selective diffusion in living organisms, *J. Gen. Physiol.*, 1922–23, v, 231.
4. Martin, E. G., The inhibitory influence of potassium chloride on the heart, and the effect of variations of temperature upon this inhibition and upon vagus inhibition, *Am. J. Physiol.*, 1898, ii, 47.
5. Armstrong, P. B., Determination of the pH of developing *Fundulus* eggs, *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 146.
6. Bodine, J. H., The action of Na, K, and Ca chlorides on the egg of *Fundulus*, *Proc. Nat. Acad. Sc.*, 1927, xiii, 698.