Effects of Na-salicylate upon the Cleavage of Sea Urchin Eggs

By

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(Received June 17, 1959)

The effect of Na–salicylate upon living matter has not been fully analyzed yet from the physiological point of view, in contrast to its common use as an antiseptic agent. A few workers (SMITH and JEFFREY, '56; SMITH, '57; and SPROULL, '57) have reported that Na–salicylate increases oxygen uptake, depletes the glycogen content with the accumulation of lactic acid and without formation of energy rich phosphate bonds. These experiments used rat diaphragm, pieces of small intestines, slices of brain and liver for living organisms. As far as these characteristics are concerned, Na–salicylate bears much resemblance to uncoupling agents of the oxidative phosphorylation such as 2, 4–dinitrophenol (DNP) and thyroxine, so it is regarded as similar to such agents. The effect of DNP on the cleaving eggs of sea urchins has often been investigated and it has been shown that DNP prevents the cleavage of eggs reversibly at concentrations higher than 10^{-6} M in sea water, and it also affects the metabolism of eggs in the manner characteristic of the uncoupling agent of oxidative phosphorylation. It was noticed that the inhibitory effect of DNP upon the cleavage could be removed by replacing the eggs in normal sea water, where cleavage resumed and proceeded quite normally in the eggs just as in newly fertilized eggs (MOTOMURA, '47; KITAZUME, unpublished). The present work has been carried out along the same line using Na–salicylate instead of DNP, and special attention has been paid to the rehabilitation process which has not been referred to in the previous works. In the present work, first the lowest concentration of Na–salicylate in the sea water which is enough to inhibit the cleavage of eggs has been determined and the recovery by washing the eggs with normal sea water has been tested. Then, the changes in the amount of O_2–uptake, of reducing sugars, of lactic acid accumulation, of adenosine-triphosphate (ATP) and in the activity of ATPase have been measured. These experiments have been carried out in July 1956 and 1958 at Seto Marine Biological Station of Kyoto University.

Materials and Methods in General

The eggs of *Heliocidaris crassispina*, obtained by means of the usual KCl method, were washed through several changes of filtered sea water and transferred

to a large glass vessel, in which they were inseminated and left to develop. The quantity of sperm added was so little as compared with eggs that it was substantially negligible. As one batch of eggs was not large enough for the chemical analyses in each series of experiments, eggs from a few batches were mixed and provided for sampling. Although almost homogeneous egg suspension could be obtained by stirring the sea water in the vessel, values obtained from quantitative analyses in each series of experiments were given per unit of dry weight or nitrogen content of the materials used. To determine the dry weight of the materials, eggs were dried in an oven at 90°C for 8 hours and the mean weight was calculated from these measurements. All the experiments were carried out at 22° to 24°C.

Experiments

1. *Inhibitory Effect of Na–salicylate upon Cleavage.*

   Methods: Immediately after the fertilization membrane was elevated, the eggs were transferred into the sea water containing Na–salicylate at various concentrations ranging from 0 to 10^{-1} M and left for two hours. After this treatment, eggs were washed through several changes of filtered sea water free of Na–salicylate, then resuspended in it for several hours and they were repeatedly brought under the microscope at short intervals to examine the rate of cleaved eggs.

   Results: Fertilized eggs in normal sea water accomplished the first cleavage within 45 minutes under the conditions of the present experiment but cleavage was inhibited in those in the sea water containing Na–salicylate at concentrations higher than 2×10^{-2} M and no cleaved egg was found within at least two hours. At concentrations lower than this, inhibition of cleavage was incomplete; at concentrations less than 10^{-3} M the cleavage was retarded slightly and all eggs cleaved within at least 60 minutes after fertilization, at 10^{-2} M inhibition was incomplete and 20–50% of the eggs remained uncleaved at the end of the second hour. At 2×10^{-2} and 5×10^{-2} M cleavage was completely inhibited. The shape of eggs remained unaltered

Table 1. Inhibitory effect of Na–salicylate upon the cleavage of
sea urchin eggs and recovery of the cleavage after washing.

Concentration of Na–salicylate in sea water	Shape of eggs	Cleavage	Cleavage after washing	Time needed for recovery
0 (control)	———	+	———	———
10^{-4} M	normal	+	———	———
10^{-3} M	,,	+	———	———
10^{-2} M	,,	±	+	30–45 min.
2×10^{-2} M	,,	–	+	30–75 min.
5×10^{-2} M	,,	–	+	,,
10^{-1} M	small droplets were formed	–	+	1.5–2 hrs.

at these concentrations while at 10^{-1} M blisters were formed on the surface of the eggs and small droplets were often observed in the space between the fertilization membrane and the egg surface, this feature resembling those of eggs intoxicated with monoiodo-acetic acid or mercury benzoate.

By washing through several changes of the sea water, eggs recovered from the inhibitory effect of Na–salicylate upon cleavage as in the case of DNP intoxication; the cleavage of eggs resumed and proceeded quite normally through succesive cell generations. However, the time needed for this recovery was correlated to the concentration of Na–salicylate in the sea water; at 10^{-2} M the time between the washing and the first cleavage was 30–40 minutes (not longer than the time from fertilization to the cleavage in the normally developing eggs), at 2×10^{-2} M and 5×10^{-2} M the time spread from 30–75 minutes, at 10^{-1} M it took 1.5–2 hours but the recovery rate reached 100% at the end of the 2nd hour after washing. The results obtained in this experiment are summarized in Table 1.

2. *Oxygen Uptake.*

Methods: The amount of O_2–uptake was estimated by means of the usual Warburg manometric method; the main chamber of the vessel contained the sea water buffered with 1/250 M glycine (final concentration) at pH 7.5–8.0 in which eggs fertilized immediately before were suspended, the center well was filled with 20% KOH solution and side arm with sea water containing Na–salicylate at respective concentrations. When temperature and vapour pressure in the vessel were stabilized the content of the side arm was tipped into the main chamber. The final concentration of Na–salicylate in the medium of the egg suspension was 0, 10^{-3} M, 10^{-2} M, 10^{-1} M respectively. The vessels were kept at 24°C throughout the experiment. As soon as the estimation of O_2–uptake was finished, eggs were examined under the microscope.

Results: As illustrated in Fig. 1, the fertilized egg consumed 66 μl of O_2 per 100 mg dry weight during 120 minutes, that is 3.6 times as high as that consumed by unfertilized eggs. The effect of Na–salicylate upon O_2–uptake of fertilized eggs varied according to the concentration of the agent in the medium. At the concentration of 10^{-3} M O_2–uptake increased slightly more than that of the control, notwithstanding that no inhibiting effect upon cleavage was recognized. At 10^{-2} M, where the cleavage was almost completely inhibited, the amount of O_2–uptake became 40% higher than that of the control. On the contrary, though, at 10^{-1} M it decreased to less than 20% of the control. The effect of this agent upon O_2–uptake of unfertilized eggs was negligible at 10^{-1} M.

3. *Amount of Reducing Sugars.*

Methods: Eggs were fixed with 5% trichloro-acetic acid (TCA) and homogenized in it. The total amount of the acid soluble carbohydrate extracted into the medium was measured by means of the anthrone method developed by KAHAN ('53) and it was regarded as an approximation of the amount of acid-extractable reducing sugars in the eggs.
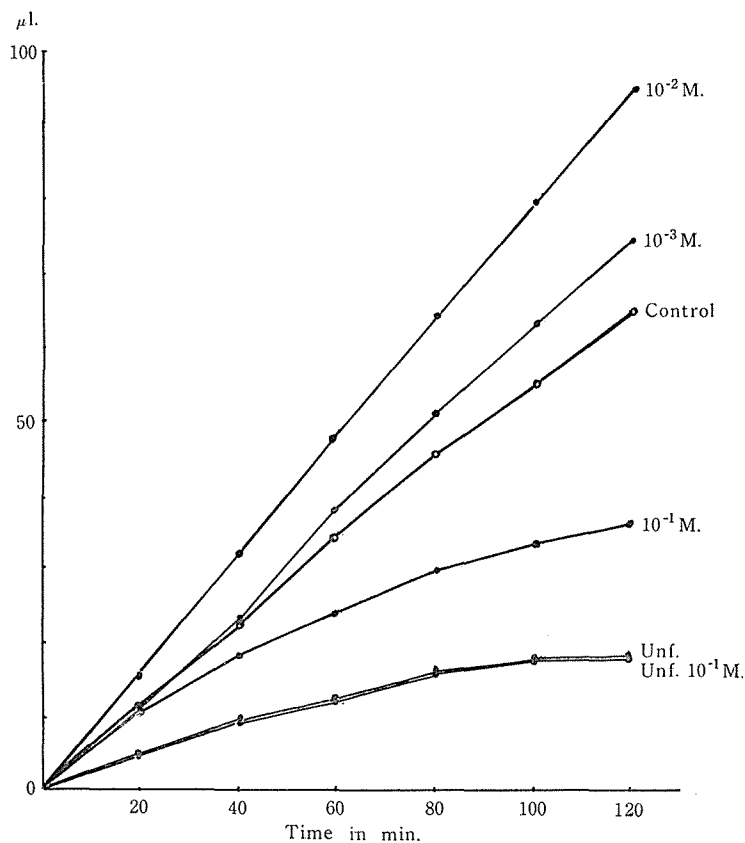
Fig. 1. Effect of Na–salicylate upon O_2–uptake of sea urchin eggs.
μl per 100 mg dry weight.

For materials, unfertilized eggs, eggs in some early stages of the normal development and those treated with 10^{-2} M Na–salicylate in the sea water were used. Under the condition of the present experiment, eggs suspended in the Na–salicylate sea water immediately after fertilization remained uncleaved at least for five hours and cleavage commenced when the eggs were washed and transferred into normal sea water at the end of the fifth hour, while the eggs fertilized and suspended in the normal sea water accomplished the first cleavage within one hour after insemination and several cleavages succeeded thereafter in the following five hours.

Sampling of the materials was performed with unfertilized eggs, with eggs immediately after the fertilization membrane was elevated and with normally developing eggs after 30 minutes, 1 hour, 3 hours, 5 hours and 6 hours from insemination respectively. Sampling of the treated materials which were suspended in the Na–salicylate sea water immediately after fertilization, was performed with eggs

treated for 30 minutes, 1 hour, 3 hours, 5 hours and those resuspended for one hour in the normal sea water after 5 hours of the treatment.

   Results: The amount of reducing sugars in the normally developing eggs decreased rapidly after fertilization and reached 70% of the unfertilized eggs within 60 minutes, then increased gradually through several cleavages in the following 5 hours. In the eggs suspended in the Na–salicylate sea water, it decreased more
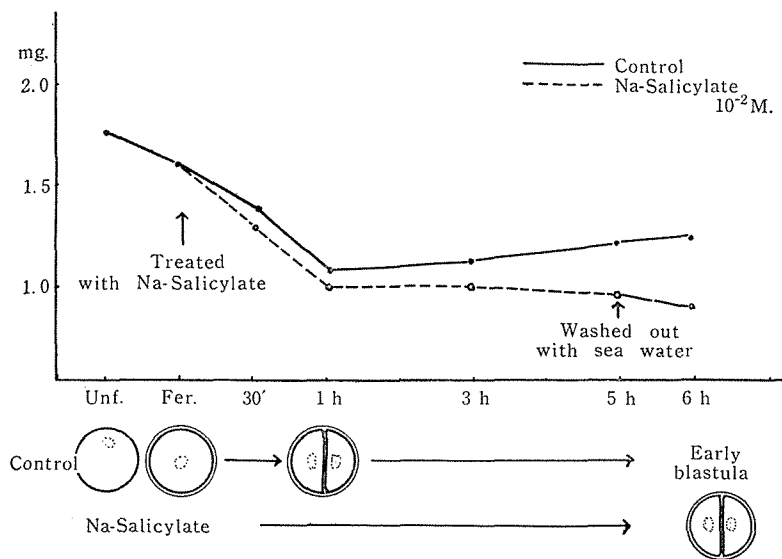

Fig. 2. Effect of Na–salicylate upon the reducing sugar content.
        mg per 50 mg dry weight.

rapidly than in the normal eggs in the first hour but it remained almost unchanged thereafter for 4 hours. When the eggs were washed and resuspended in normal sea water at the end of the fifth hour, the amount of reducing sugars began to decrease with the onset of cleavage just as in newly fertilized eggs.

4. *Lactic Acid Accumulation.*

   Methods: The amount of lactic acid accumulated in the eggs was estimated with BARKER and SUMMERSON's method ('41). The procedures of preparing samples were the same as those in the glycogen analysis mentioned above, but in the present analysis p–hydroxy-diphenyl was used instead of anthrone and H_2SO_4 was added to the reaction medium in an ice chilled water bath to avoid local heating of the medium.

   For materials, both normally developing eggs and those suspended in the Na–salicylate sea water at concentration of 10^{-2} M were used.

   Results: As shown in Table 2, unfertilized eggs contained 13 μg of lactic acid

Table 2. Effect of Na–salicylate upon the accumulation
of lactic acid. μg per 50 mg dry weight.

	Unf.	Fert.	30 min.	1 hr.	2 hrs.	3 hrs.
Control	13.1	10.5	10.5	8.5	10.2	10.5
Na–salicylate (10⁻² M)	—	—	11.5	12.7	13.7	16.5

per 50 mg dry weight. In the eggs shortly after fertilization, the amount of lactic acid decreased remarkably, it reached a minimum after 60 minutes and gradually increased thereafter. In fertilized eggs suspended in the Na–salicylate sea water, in which the cleavage of eggs was inhibited, the amount of lactic acid was slightly more than the level in fertilized eggs prior to the treatment, then it increased gradually and at the end of the 3rd hour it exceeded the level of the unfertilized eggs by 25%.

5. *ATP Content.*

Methods: Essentially the same method as that of LePage ('49) was used to fractionate the labile phosphate compounds. Eggs were homogenized with 10% TCA in an ice chilled water bath, the supernatant of the homogenate was precipitated with Ba–acetate regulated at pH 8.2 using KOH and then resolved in dilute HCl; this procedure was repeated twice. The excess of Ba ions was removed by adding 0.05 N H_2SO_4. The sample thus prepared was divided into two parts, one part was hydrolyzed with N HCl at 100°C for 7 minutes to release labile phosphate from the compounds. The inorganic phosphate in both samples, hydrolyzed and unhydrolyzed, was estimated respectively following ALLEN's method ('40), but 5 N H_2SO_4 was used instead of 60% $HClO_4$ to avoid precipitating $-ClO_4$ and K ion in the samples.

Difference in the amount of phosphate between the two samples was regarded as the amount of the labile phosphate (Δ 7P) included in the eggs. With the method used in the present experiment it is impossible to distinguish each component of the labile phosphate, however, it is of no importance to discriminate ATP from ADT, and the total amount of the labile phosphate was taken to be the amount of ATP for convenience in the present work.

Results: The amount of ATP in the unfertilized eggs was immeasurably small with the method used in the present experiment (Fig. 3). After fertilization ATP accumulated rapidly and reached a maximum at the end of the first hour when most of eggs accomplished the first cleavage. Then it decreased gradually with the lapse of time. In eggs suspended in 5×10^{-2} M Na–salicylate sea water, in which the cleavage of eggs was completely inhibited, ATP was accumulated during the first 30 minutes at the same rate or slightly lower rate than in the normally developing eggs, but it decreased rapidly in the next 30 minutes to about 80% of the amount accumulated in the normally developing eggs at the end of the first hour and thereafter it remained almost unchanged as long as the eggs were suspended in
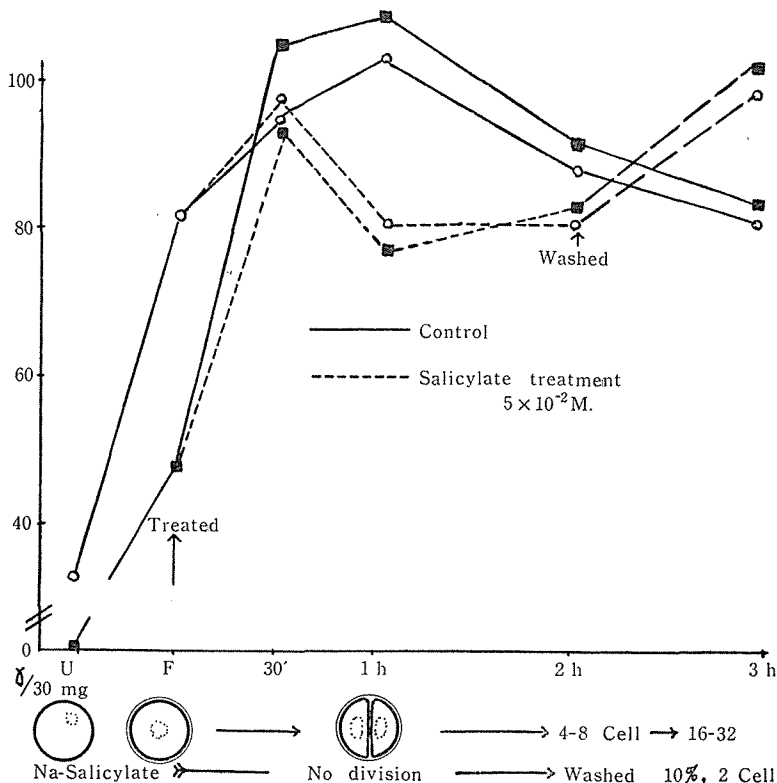
Fig. 3. Effect of Na-salicylate upon the amount of Adenosine-
triphosphate (ATP). μg per 30 mg dry weight.

the Na–salicylate sea water. After 3 hours, the eggs were washed and transferred to normal sea water. Then accumulation of ATP was resumed and restored to the level of the first 30 minutes within one hour, although only 10% of the eggs cleaved in the meantime.

6. ATPase Activity.

Methods: The activity of the ATP–dephosphorylating enzyme system was tested by measuring inorganic phosphate liberated from the ATP used for substrate. As the enzyme source, whole egg homogenate, one part of eggs homogenized one hour after fertilization with 5 parts of normal sea water in an ice water bath, was used. To 1 cc of the homogenate thus prepared, 1 cc of 1.6×10^{-3} M Na$_3$–ATP (Wako Chemical Co.) and 2 cc of 0.2 M Tris. buffer (pH 7.2) were added. In the present experiment it was not necessary to add Mg ion because the concentration of Mg ions initially introduced from the sea water used for homogenization of eggs was sufficiently high in the reaction medium being at 10^{-3} M or more. The reaction

medium was kept at 30°C for 30 minutes and then the enzymatic action was
stopped by adding 1 cc of 50% TCA, and the liberated inorganic phosphate was
estimated with ALLEN's method described above.

The experiments were carried out with the following five samples : 1) the
substrate only, without homogenate, 2) the reaction medium in which the enzyme
activity was abolished by heating the homogenate at 100°C for 5 minutes, 3) the
reaction medium alone, without addition of agent, 4) the reaction medium, to which
DNP was added at the final concentration of 10^{-3} M, and 5) the same, with 5×10^{-2} M
Na–salicylate instead of DNP.

Results : In both experiments 1 and 2, an almost equal amount of inorganic
phosphate was detected notwithstanding that the enzyme was not present in sample
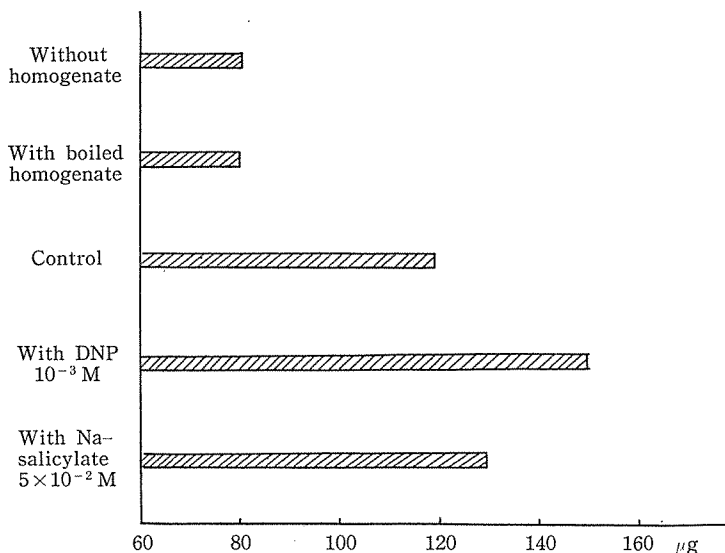1 and the activity of the enzyme was abolished in sample 2. So the inorganic

Fig. 4. Effect of Na–salicylate upon the ATPase.
μg liberated inorganic phosphate.

phosphate liberated from the substrate ATP was regarded as a consequence of
spontaneous splitting of ATP under the conditions of the present experiments.
Therefore, the amount of inorganic phosphate in the foregoing experiments was
substracted from the total amount of inorganic phosphate in the three experiments
respectively to estimate the amount of phosphate set free by the action of ATPase.
As illustrated in Fig. 4, the activity of ATPase was stimulated with 5×10^{-2} M
Na–salicylate by 25% and remarkably with 10^{-3} M DNP by 70% over the intrinsic
activity.

Discussion

The effect of Na–salicylate upon cleavage of sea urchin eggs quite resembles that of DNP in many respects. At appropriate concentration it arrests the cleavage of eggs reversibly, stimulates O_2–uptake and accelerates consumption of reducing sugars in a period following fertilization. With Na–salicylate more concentrated solutions are needed than DNP to make effects of the same order. For example, the lowest concentration of Na–salicylate which brings about complete but reversible blocking of the cleavage of eggs is 2,000 times higher than that of DNP. This difference in effectiveness would not depend upon the difference in materials used in the experiments, since cleavage of eggs in different species of sea urchins is inhibited with DNP at almost the same concentration (MOTOMURA, '47 ; CLOWES *et al.*, '50 ; KITAZUME, unpublished).

Changes in the amount of reducing sugars in cleaving eggs are suggestive. With the method used in the present work, only the total amount of all the acid soluble reducing sugars in the eggs was analyzed but not glycogen or glucose and other sugars separately. The early developmental period of the eggs can be divided into two successive phases with regard to the metabolism of sugars ; the first phase (that is the first 60 minutes after fertilization), in which the amount of reducing sugars decreases rapidly and the 2nd phase in which it increases very gradually. Since most of the carbohydrate included in unfertilized eggs of sea urchins is considered to be glycogen or glucose (HUTCHENS *et al.*, '42), decrease of reducing sugar content in the first phase may be due chiefly to breakdown of these sub-stances. In many works it is recognized that DNP stimulates the glycolysis and the results obtained in the present work also indicates the very similar effect of Na–salicylate at least in the 1st phase of sugar metabolism. In the 2nd phase, Na–salicylate seems to arrest the accumulation of reducing sugars in the eggs as long as the eggs are placed in Na–salicylate sea water. However, it cannot be considered that Na–salicylate inhibits formation of reducing sugars, because we only know the amount of reducing sugars in total, and quantitative changes in each kind of the sugars cannot be discriminated by the method employed in the present work. At present, we have no direct evidence to infer the metabolic pathway through which the increase of sugar content is yielded in the 2nd phase. But there would be possibilities that sugars may be formed from some substances derived from lipids or amino acids but not from intermediates of glycolysis. Data obtained in Na–salicylate treatment suggest that there are two reactions operating in the early developmental period with regard to sugar metabolism, of which one may be glycolysis and the other one production of sugars through ways other than a glycolytic pathway. In the 1st phase, the quantity of sugars broken down through the glycolytic way would exceed that of formed sugars resulting in a decrease in the sugar content, and in the 2nd phase the relation between the two reactions would be the reverse. Under the influence of Na–salicylate, the amount of sugars formed and the amount of sugar depleted through a glycolytic pathway compensated for each

other, leaving the sugar content unchanged in the 2nd phase, since the glycolysis is accelerated by Na–salicylate.

Lactic acid is already accumulated to some extent in unfertilized eggs. Its amount decreases shortly after fertilization, becomes lowest at the end of the first 60 minutes and then is restored to the level of fertilized eggs. (AKETA ('57) found a sudden increase of lactic acid content of sea urchin eggs at fertilization. This temporary increase will be overlooked in the present work.). In eggs treated with Na–salicylate, decrease in the amount of lactic acid shortly after fertilization is less marked than in the control eggs and with the lapse of time lactic acid is accumulated continually beyond the level of the lactic acid content of the unfertilized eggs. Such an accumulation of lactic acid accompanied by rapid depletion of glycogen content has been reported by SMITH and JEFFREY ('56) in tissues intoxicated by Na–salicylate. This continuous accumulation of lactic acid might be considered to indicate the prevalence of rapid and uninterrupted glycolysis in eggs treated with Na–salicylate.

HULTIN ('57) reported that the ATP content of sea urchin eggs gradually decreased as cleavage of eggs proceeded. KRAHL ('50) stated that the ATP content remained practically constant in developing eggs of sea urchin as in amphibian embryos (referring to LINDBERG's work, in which the rate of incorporation of labelled P into the ATP fraction of sea urchin eggs is much higher in fertilized eggs than in unfertilized eggs). In the present work, however, ATP content of control eggs increases rapidly after fertilization in harmony with rapid consumption of glycogen. This datum does not coincide with that obtained by HULTIN but it may depend on differences either in material or method of analysis. Under the influence of Na–salicylate such an increase in ATP concentration also occurs in the first 30 minutes of fertilization at almost the same rate as that of the control, but in the next 30 minutes it decreases to a level 20% lower than that of the control, at which it remains continually unchanged while the eggs are kept in the medium containing Na–salicylate where cleavage of eggs is completely arrested. The activity of latent ATPase is found to be stimulated with Na–salicylate as with DNP. The sudden increase of ATP content in the eggs treated with the agent seems to be inconsistent with this fact; however, this conflict would be interpreted as that ATP may be produced in the eggs during the latent time before Na–salicylate becomes effective upon ATPase included in the eggs.

Recently, MESSER ('58) investigated the effect of some agents such as barbiturate, salicylate, chloropromazine, 2,4–DNP and cyanide upon glutamine synthesis and found that only Na–salicylate has the same effect as DNP. The effect of Na–salicylate on the cleaving eggs of sea urchins coincides with that of DNP in every respect, and we have ample evidence to consider Na–salicylate to be one of the uncoupling agents of oxidative phosphorylation.

Summary

1.　The effect of Na–salicylate upon the cleavage of the sea urchin eggs,

Heliocidaris crassispina, was investigated. The cleavage of the eggs was inhibited with Na-salicylate at concentration higher than 10^{-2} M. The inhibiting effect was reversible by washing with normal sea water, cleavage commenced and proceeded quite normally in those eggs just as in newly fertilized eggs.

2. O_2-uptake was slightly stimulated with the agent at 10^{-3} M notwithstanding that no inhibiting effect upon the cleavage was apparent at this concentration. At 10^{-2} M O_2-uptake reached a peak at about 40% more than that of control at the end of 2 hours after treatment. On the contrary, O_2-uptake was inhibited at 10^{-1} M.

3. The amount of reducing sugar in the eggs treated with 10^{-2} M agent decreased more rapidly than in the normal eggs within the first 30-60 minutes but it remained almost unchanged thereafter as long as the eggs were treated with the agent sea water. After washing the eggs with normal sea water, the sugar concentration began to decrease with the onset of cleavage. The amount of lactic acid accumulated gradually after treatment with 10^{-2} M agent.

4. The amount of ATP in the eggs suspended in 5×10^{-2} M Na-salicylate (in which the cleavage of the eggs was completely inhibited) increased within the first 30 minutes at the same rate as in normally developing eggs and it decreased rapidly in the next 30 minutes to about 80% of the amount accumulated in the normally developing eggs and thereafter it remained almost unchanged. When the effect of Na-salicylate was removed with normal sea water, the accumulation of ATP was resumed with the onset of the cleavage of eggs. The activity of ATPase was stimulated with 5×10^{-2} M Na-salicylate by 25% over the initial activity.

5. Effects of Na-salicylate upon the early cleavage of sea urchin eggs are discussed in comparison with that of DNP.

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