

THE EFFECTS OF URETHANE AND CHLORAL HYDRATE ON
OXYGEN CONSUMPTION AND CELL DIVISION IN THE EGG
OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

BY KENNETH C. FISHER AND R. J. HENRY

(From *The Marine Biological Laboratory, Woods Hole,
Massachusetts*)

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INTRODUCTION

A quantitative examination of the inhibition of oxygen consumption by urethane in the yeast cell was made recently by Fisher and Stern (1942). These authors concluded that the normal oxygen consumption of yeast was due to the activities of two independent and parallel chains of chemical reactions which differed in their sensitivity toward the narcotic, only one being affected by low concentrations though both were inhibited at higher concentrations. It was shown that inhibition of cell division by urethane in this cell was closely correlated with inhibition of one of the two systems suggested by the respiration experiments. From these data, and others in the literature, the inference was drawn that in yeast, luminous bacteria, and brain cells, narcotics effect a quantitative separation of two respiratory systems. In two of the three cases the separation may be qualitatively distinct as well, for the activities of cell division in one instance, and of light production in the other, appeared to be associated with only one of the two systems. A fractionation of the normal respiration into an "activity" and a "resting" portion, employing these terms in the sense introduced by Stannard (1941), seems to be accomplished by the narcotic. The significance of these findings to narcosis in general has been reviewed briefly by Fisher (1942).

In at least two other cases, frog muscle (Stannard, 1941) and the sea urchin egg (for references see Ballentine, 1940; and Korr, 1937), fractionations of the oxygen consumption similar to that described above for narcotics have been suggested. It was deemed of importance to determine the effects of narcotics in one or more of these cases. The present paper deals with such observations on the egg of the sea urchin. This preparation is of particular interest since a function, cell division, can be initiated at will by fertilization. Two different narcotic agents have been employed, ethyl carbamate (urethane) and chloral hydrate. The effects of these substances on the consumption of oxygen in both the fertilized and unfertilized cells have been determined, as well as the effects on cell division in the fertilized egg.

Materials and Methods

Eggs and sperm of the sea urchin, *Arbacia punctulata*, were obtained essentially as described by Just (1939). The eggs were concentrated by very low speed centrifugation and were then used as a suspension in sea water, the mixture containing 5 to 10 per cent by volume of eggs in the case of fertilized cells or 10 to 20 per cent of unfertilized cells. The percentage of eggs fertilized was uniformly high, averaging 90. The unfertilized eggs used were routinely examined to determine that unintentional fertilization had not taken place.

Oxygen consumption was measured by Warburg's direct method (Dixon, 1934) with air in the gas phase.¹ The vessels were shaken approximately 70 times per minute through an arc of 8 cm. Urethane was added as a solution in sea water directly to the egg suspension at the time the vessels were made up. Chloral hydrate in sea water was tipped into the egg suspension from the onset at the end of the equilibration period. This procedure minimizes the inaccuracy which arises from the fact that chloral hydrate solutions decompose gradually liberating a gas which is not absorbed by the alkali in the Warburg vessels. The gas produced in these experiments became a significant factor only when the chloral hydrate concentration was raised to approximately 0.1 to 0.2 M. Data at higher concentrations than these will not be reported. The respiratory experiments were performed at 25°C. and as a rule were terminated within 3 hours after collecting the eggs.

Since Smith and Clowes (1924 *a*) have shown that the rate of cell division is relatively independent of pH over the range 6.0-8.3 no attempt was made to control this factor rigidly in the present investigation. Actually it was always within the range of 7.5-8.3, sodium hydroxide being added to the chloral hydrate solutions to neutralize the free acid which was present.

To determine the effect of the inhibitors on cell division, approximately equal numbers of eggs were placed in each of several different concentrations of the narcotics considerably before the first division had occurred. Development was allowed to proceed until, in the controls without inhibitor, the 16 and 32 cell stage had been reached. The time required was approximately 3 hours at the temperature of these experiments, 24°C. Division was then stopped in all of the experimental solutions by the addition of sufficient formalin to make its concentration 0.1 per cent. An index of the average velocity of cell division over the period of exposure to the narcotic was obtained by the method described by Smith and Clowes (1924 *b*). Each value to be reported is based upon the examination of at least one hundred eggs in each solution. In the present paper the velocity of cell division determined in this way will be expressed as a percentage of the uninhibited control rate. The average difference between duplicate controls is of the order of 1 per cent.

RESULTS

Exclusive of preliminary observations establishing the appropriate range of inhibitor concentration, fertilized sea urchin eggs were subjected to a series of

¹ The rate of diffusion of oxygen from air was adequate as evidenced by the fact that the rate of oxygen consumption in air and in oxygen were found to be identical.

urethane concentrations in five complete experiments. The consequent reductions in the rate of oxygen consumption and of cell division in a typical experiment are shown in Fig. 1. The respiratory data are plotted to test the possibility of describing the inhibitory effects in terms of the law of mass action (*cf.* Fisher and Stern, 1942). The required straight line is not found and consequently the data cannot be described by a simple equation derived from the

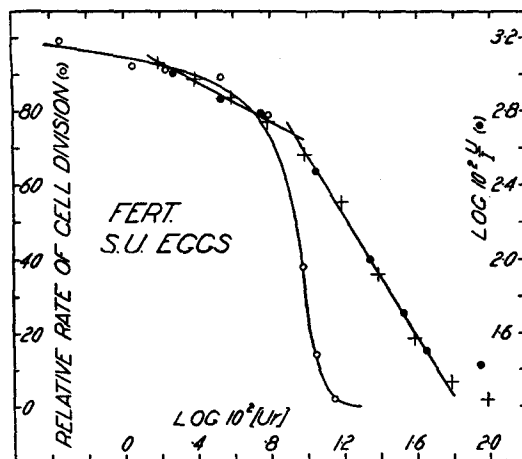


FIG. 1. The effect of different concentrations of ethyl carbamate on the rate of cell division (open circles) and on the rate of oxygen consumption (solid circles) in the fertilized sea urchin egg. U , the "uninhibited" oxygen consumption is the consumption which remains in the presence of a given molar concentration ($[Ur]$) of urethane. I , the "inhibited" oxygen consumption is the difference between U and the normal oxygen consumption. If the respiratory data are capable of description in terms of the law of mass action, it should be found that $\log \frac{U}{I} = \log K - a \log [Ur]$, K and a being constants (*cf.* Fisher and Stern, 1942); *i.e.*, $\log \frac{U}{I}$ plotted as a function of $\log [Ur]$ should give a straight line whose slope is a .

mass law. However, with the exception of the determination at the highest concentration, it is apparent that these data, as plotted, conform closely to two intersecting straight lines. The intersection occurs at a urethane concentration of approximately 0.1 M ($\log 10^2 [Ur] = 1.0$).

Fig. 1 also illustrates the effect of urethane on the rate of cell division. In 0.1 M urethane cell division is practically stopped, although at that concentration the rate of oxygen consumption has only been reduced to about 75 per cent of the normal.

Typical data (single experiment) obtained for *chloral hydrate*, the second

narcotic agent investigated, are given in Fig. 2. Again the respiration observations have been plotted to test the applicability of the mass law. As in the case of urethane they do not at all approximate a single straight line, and consequently cannot be accurately described by a single equation derived from the law of mass action. Instead they too conform reasonably well to two intersecting straight lines. The intersection or bend occurs at a chloral hydrate concentration of approximately 0.0045 M ($\log [C.H.] 10^4 = 1.65$). This concentration, as shown in the figure, is sufficient to stop cell division although it leaves 45 per cent ($\log \frac{U}{I} 10^2 = 1.91$) of the normal rate of oxygen consump-

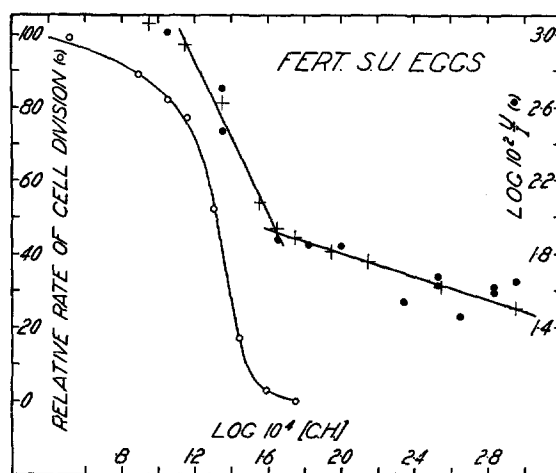


FIG. 2. The effect of chloral hydrate on oxygen consumption and cell division in the fertilized sea urchin egg. Symbols as in Fig. 1.

tion. The experiments with both urethane and chloral hydrate thus exhibit clearly the characteristic ability of narcotics to depress cellular functions unequally. In each case cell division is much more severely affected than is oxygen consumption.

Cognizance must be taken of two factors which might distort an otherwise simple relation between concentration and effect, thus producing as an artifact the form of the respiratory data seen in Figs. 1 and 2. These are (1) irreversible damage, and (2) uncertainty with regard to the equilibrium concentration of the inhibitor due to the uptake of inhibitor by the cells. As judged by the ability to resume normal cleavage upon removal of the inhibitor an exposure longer than 70 minutes to a 0.34 M solution of urethane was required to cause irreversible damage. Complete reversibility of the effects of chloral hydrate was observed after an immersion for 1 hour in a 0.022 M solution and after 30

minutes in a 0.044 M solution. Since the intersections in the plotted respiratory data were found at 0.1 M urethane and at 0.0045 M chloral hydrate respectively they cannot be due in any way to the onset of irreversible changes. The suppression of the oxygen consumption by various concentrations of either inhibitor was constant when the ratio of egg volume to narcotic volume was varied from 1:10 to 1:60. It follows that the quantity of inhibitor bound by the eggs was insignificantly small.

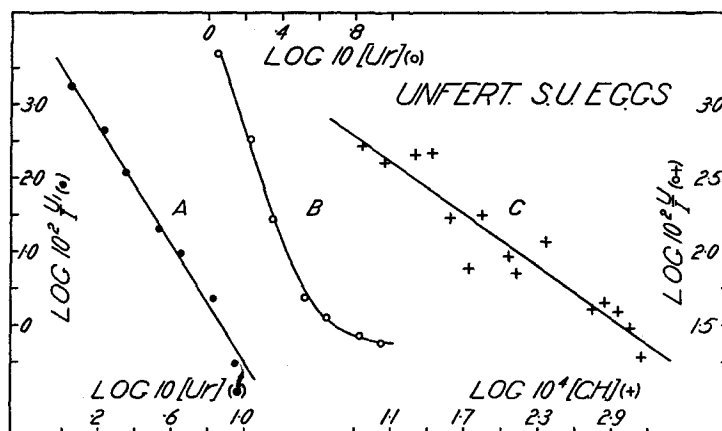


FIG. 3. The effects of urethane and of chloral hydrate on the consumption of oxygen by unfertilized sea urchin eggs. The method of plotting is the same as that described for the respiratory data of Fig. 1. The average observations from six separate experiments on urethane are shown in B; the same data are plotted in A after account is taken of an inhibitor-stable respiration amounting to 19¼ per cent of the normal oxygen consumption. Line C typifies the results of the experiments with chloral hydrate. In this case the observations from a single experiment rather than from an average are quoted, in order to illustrate the variability which may occur. See text for details.

Since neither damage nor uncertainty regarding the narcotic concentration is concerned in determining the relation between concentration and effect, it seems that the relations observed must be ascribed to the normal cellular respiratory systems. A striking difference between the actions of the two inhibitors will be noted. Whereas the slope of the line describing the effect of urethane at the higher concentrations is greater than that of the line describing the effect at low concentrations, the exact opposite is true of the lines for chloral hydrate.

The characteristics of the inhibition of oxygen consumption in the unfertilized eggs by the two narcotics are illustrated in Fig. 3. It will be recalled (Fig. 1) that in the fertilized egg 0.1 M urethane reduces the consumption of oxygen to 75 per cent of the normal value and stops cell division. That concentration

in the unfertilized egg has practically no effect (line B, Fig. 3), at most reducing the respiration by only 3 per cent (to $\log \frac{U}{I} 10^2 = 3.5$). The range of urethane concentrations producing graded inhibitions of respiration in the unfertilized egg thus extends upwards from the concentration which suffices to prevent cell division. In contrast the effective concentration range for chloral hydrate is practically identical for the fertilized (Fig. 2) and unfertilized egg (line C, Fig. 3).

Lines B and C of Fig. 3 are straight over most of the range of inhibition which each covers. The slopes of these linear portions are different. They are compared in Table I with the slopes of the intersecting lines found in Figs. 1 and 2.

With each narcotic the value observed for the unfertilized egg is not equal to either of the two observed for the fertilized egg but it is distinctly closer to one of them than to the other. That one in both cases is the value character-

TABLE I
The Apparent Values of a

	Fertilized egg		Unfertilized egg
	Low concentration	High concentration	
Urethane.....	0.53	1.6	3.8
Chloral hydrate.....	2.1	0.32	0.6

istic of the high range of inhibitor concentrations. It applies, as has been noted earlier, to the inhibition which is produced by concentrations greater than those which block cell division.

The points for urethane in Fig. 3B tend to approach asymptotically a level at approximately 19 per cent of the normal rate of oxygen consumption ($\log \frac{U}{I} 10^2 = 1.3$). It is thus suggested that this 19 per cent is urethane-stable.

A straight line is to be expected on the axes used here only when the respiration is completely sensitive to the inhibiting agent. Where a stable fraction exists U (as defined for Figs. 1 and 2) minus this fraction should be employed rather than U alone. The new quantity may be designated U_1 and the results of using it, considering $19\frac{1}{4}$ per cent of the normal oxygen consumption to be unaffected by urethane, are illustrated by line A of Fig. 3. The points conform closely to a straight line, indicating that the mass law provides an adequate description of the effects of urethane on the part of the respiration which is sensitive to this inhibitor. Line B was calculated using the constants obtained from line A and taking into account the narcotic-stable fraction.

The experiments with chloral hydrate do not reveal any narcotic-stable oxygen consumption. This is undoubtedly due to the fact that the gas production referred to earlier prevented the use of the high concentrations which would be necessary to demonstrate it.

Cell Division

For comparison with the data already presented the observations on cell division which were included in Figs. 1 and 2 respectively, appear again in Fig. 4 where they have been plotted to test the applicability of the law of mass action. It is apparent that in neither case is the required straight line found. Further

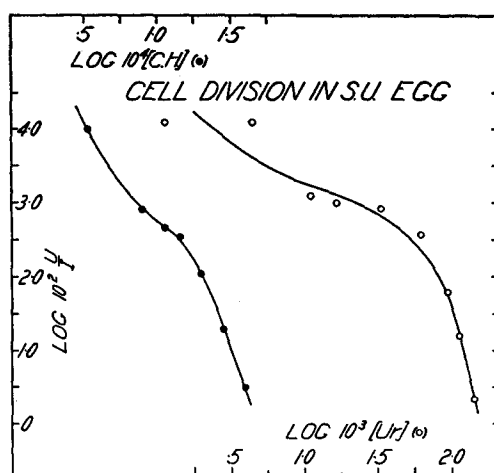


FIG. 4. The possibility is tested here of describing the data for cell division from Figs. 1 and 2 by an expression of the law of mass action. See text for further details.

experiments are necessary in this connection since it is possible that the shape of the curves may be affected by the criterion of rate of cell division which was used.

The general conclusions to be drawn from the foregoing observations must take into account the following principal facts: (1) In unfertilized eggs which do not divide the mass law plot of the respiratory data shows only one line, while (2) in the fertilized egg which does divide two intersecting lines appear. (3) Complete inhibition of one function, cell division, occurs at narcotic concentrations which cause a relatively smaller effect on another function, oxygen consumption. (4) Where two intersecting lines are found in the mass law plot, the slope of the line at the lower inhibitor concentrations is greater than that of the line at the higher concentrations for one narcotic, but is just the opposite for the other. (5) For both narcotics the slope of the line for un-

fertilized eggs tends to approach in value the slope of the line covering the higher concentration range of the same inhibitor for the fertilized eggs.

It will be noted at once from point (1) above, that the effects of narcotics in the unfertilized egg can be completely accounted for if there exists only a single site of narcotic action in those cells. However, on the basis of a single site it seems quite impossible to account in a reasonable manner for the observations on the fertilized egg (points (2) to (5)). If two such sites were involved, the next simplest possibility, they might be in series in a single chain of respiratory reactions, or each might be in one of two parallel chains which together made up the total oxygen consumption of the cell. With two sites of action in either series or in parallel, and with the effect of the inhibitor at each governed by the mass law, calculation shows that it is still possible for the total respiration of the cell to give practically a straight line when plotted as in Figs. 1 and 2. As far as the immediate considerations are concerned therefore, the narcotics could conceivably act at two sites in the unfertilized as well as in the fertilized egg. The approximation to a single straight line happens, however, only when the two sites are affected over essentially the same range of concentration. If they are affected by significantly different ranges of concentration, a more likely situation, the resultant of the two effects differs very markedly from a single straight line, just as the data for the fertilized egg do in Figs. 1 and 2.

Suppose then that there are two sites in series and that they are acted upon by different ranges of inhibitor concentration. As the concentration gradually rises, the degree of inhibition will change with the concentration at a rate determined primarily by the characteristics of the more sensitive of the two sites. Let the concentration now become great enough to cause an effect at the second one. The simplest conditions which can be imagined require that the metabolism already partially inhibited at one stage shall be still further reduced at the second. It thus follows that when the concentration has been raised sufficiently to cause inhibition at both sites, the degree of inhibition must increase more rapidly with concentration thereafter, than it would if the narcotic were acting at only a single site. This is the picture given by urethane in Fig. 1.

The degree of inhibition of oxygen consumption as measured by $\log \frac{U}{I}$ increases more rapidly at concentrations above the point of intersection, than it does at concentrations below that point. The degree of inhibition with chloral hydrate on the other hand changes less rapidly with concentrations above the point of intersection than it does at concentrations below that point. While a final statement is perhaps impossible it is difficult to see how this could happen if there were two sites and these were in series. It is likewise difficult if not actually impossible at present to visualize a mechanism which would account for points (3) and (5) on the basis of two sites of action in series.

These experiments indicate in general then that there are at least two distinct

sites for the action of chloral hydrate and of urethane, but that these are not likely to be in series.

On the other hand, the properties of a parallel arrangement of the two sites are such as indicate that this arrangement would account for all of the observations recorded here. It requires that the total oxygen consumption of the cell be the sum of the oxygen consumptions by two separate parallel chains or systems of respiratory reactions. Each includes a step which is inhibited by narcotics and since these two sites are distinct there will be a separate expression of the mass law describing the action of the narcotics at each site. The form of the graph to be expected when the total oxygen consumption of such a preparation is plotted as in Figs. 1 and 2, can readily be calculated. As was stated above it may approximate a single straight line (such as was found for the unfertilized egg) or it may instead approximate two intersecting straight lines as do the data for the fertilized eggs. The parallel arrangement permits the possibility of the observations referred to under point (4) above although the series arrangement does not, as has been noted.

Two straight lines similar to those in Figs. 1 and 2 result when the affinities of the two sites for inhibitors are sufficiently different, that is, when one of the chains is appreciably inhibited by concentrations too low to affect the other. In this case the per cent inhibition of the more sensitive system increases more rapidly as the concentration is raised than does the per cent inhibition of the total oxygen consumption (= sum of oxygen consumption by the two separate chains). The data for the fertilized egg thus suggest not only that two respiratory systems in parallel are responsible for the observed oxygen consumption, but also that one of the two must be affected at significantly lower inhibitor concentrations than is the other. Cell division is inhibited more rapidly as the concentration of narcotic rises, than is the total oxygen consumption of the egg. Inhibition of cell division then, parallels inhibition of the more sensitive of the respiratory systems more closely than it does inhibition of the total oxygen consumption. It may be that the oxidative metabolism required for cell division is mediated by this chain rather than the one affected only at higher concentrations of narcotic. Because of the correlation if not actual connection between the activity of cell division and one of the chains of respiratory reactions that one, the more sensitive of the two, will henceforth be referred to as the "activity" system or chain. The term "basal" or "resting" will be applied to the remaining system.

Point (5) dealing with the slopes of the lines on the double log axes, leads to a conclusion regarding the identity of the narcotic-sensitive respiratory system active in the unfertilized egg and the basal system of the fertilized cell. When only a single site of narcotic action exists, the slope of the line on the double log axes gives directly the value of a in the expression of the mass law which describes the data. If there are two sites and two lines result when the total

oxygen consumption is used in making the double log plot, then the slope of each line may differ very appreciably (50 to 60 per cent) from the value of a which primarily determines it. The values of these slopes therefore will be referred to as "apparent" values of a to distinguish them from the true values. The slopes for the fertilized egg which are listed in Table I are apparent values while those for the unfertilized egg, since they were determined from single lines, may be true values. In general the apparent values are always less than the true values. The latter for the fertilized egg in the high concentration range must then be much closer to the values for the unfertilized egg than are the apparent values listed in the table.

If the mass law constants are such as cause the data to approximate a single line even when two parallel sites are involved, then the slope of this line, the apparent a , is nearly the average of the two separate values of a which are concerned. It is evident from Table I that the slopes for the unfertilized egg are not the average of the respective two slopes for the fertilized egg. If two sites of narcotic action exist in the unfertilized egg these cannot be the two which operate in the fertilized egg. Of the several possibilities which exist it is simplest to consider that there is only one site in the unfertilized cell so that the slope in this case gives the true a . It has already been noted that the slope in the unfertilized cell is much more similar to the true a for the basal system than it is to that of the activity system. There is a real possibility therefore that the narcotic-sensitive respiratory system of the unfertilized cell may be identical with the basal system of the fertilized one. An evaluation of the possibility requires a determination of the true a 's for the fertilized cells.

Unfortunately it is not possible to calculate from the experimental data the constants for the two expressions of the mass law required to describe the effects of each inhibitor on the two respiratory systems of the fertilized egg. They can be estimated graphically, however, by trial. Values determined in this way for the fertilized egg, along with those calculated for the unfertilized egg from the data in Fig. 3 are given in Table II. The correspondence between the true value of a for the basal system and that for the unfertilized egg is striking, suggesting strongly that the former system and the one operating in the unfertilized eggs are in fact identical.

The graphic method produces also the estimate that 40 per cent of the total oxygen consumption of the fertilized egg is mediated by the activity system, the remaining 60 per cent by the basal system.

The curves corresponding to the constants given in Table II are shown in Fig. 5. A theoretical set of data may be constructed from them (Fisher and Stern, 1942) for comparison with the experimental data. The large crosses which appear in Figs. 1 and 2 are points calculated in this way. It is evident that they describe the data in an adequate manner. The conclusion that there are two parallel respiratory systems in the fertilized egg is thus quantitatively

as well as qualitatively justifiable. In the unfertilized egg the indication is that only a single respiratory chain is present and that it is identical with the

TABLE II

A Comparison of the Values of the Mass Law Constants for the Inhibition of Oxygen Consumption in Fertilized and Unfertilized Eggs of the Sea Urchin

	"Activity" system		"Basal" system	
	Fertilized	Unfertilized	Fertilized	Unfertilized
Urethane:				
a	0.5	?	3.0	4.0
$\log K$	$\bar{1}.7$?	$\bar{2}.0$	$\bar{3}.48$
Chloral hydrate:				
a	6.0	?	0.5	0.6
$\log K$	$\bar{1}6.9$?	$\bar{1}.25$	$\bar{2}.87$

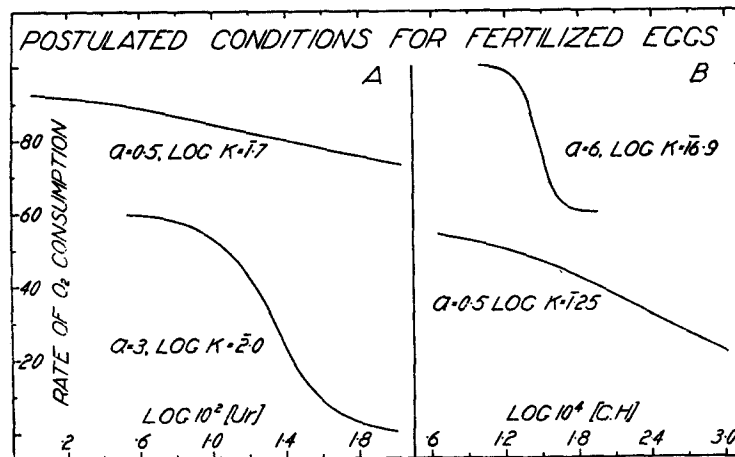


FIG. 5. *A.* The inhibition of the two postulated systems as a function of $\log [\text{Ur}]$. *B.* The inhibition of the two postulated systems as a function of $\log [\text{C.H.}]$. The upper curves in both cases refer to the inhibition of a respiratory system which will be designated as the activity system. Similarly the lower curves indicate the effect on the basal system. The normal rate of oxygen consumption is assumed to be 100 units. Forty of these are contributed by the activity system, the remainder by the basal system.

basal system. The activity system is apparently not functional until the cells have been fertilized, an additional circumstance which points to a specific relation between it and the process of cell division which is also initiated by fertilization.

In connection with the conclusions reached above, the recent observations by Krahl, Keltch, Neubeck and Clowes (1941) on the sea urchin egg are of great interest. These investigators have reported that azide will remove only 50 per cent of the total oxygen consumption of the fertilized egg. Moreover the concentration of this inhibitor required to stop cell division is just sufficient to produce the maximum arrest of oxygen consumption. The similarity between the conclusions reached above and those to be drawn from these experiments with azide are obvious and need no comment.

It will perhaps have been noted that the amount of oxygen consumed by the basal system has not been compared in the foregoing with that consumed by the unfertilized eggs. Ultimately the analysis of the metabolic changes upon fertilization must include this comparison. The result of it can hardly alter the general conclusions now reached, however, and consequently it will not be discussed in the present communication.

SUMMARY

The effects of a series of concentrations of the narcotics, ethyl carbamate and chloral hydrate, have been determined on the consumption of oxygen by fertilized and unfertilized eggs of the sea urchin *Arbacia punctulata*. In the fertilized eggs the effects of the two inhibitors on cell division were also examined. The following observations were made:

1. Assuming that the narcotic acts upon a single catalyst in the unfertilized egg the degree to which the consumption of oxygen is inhibited in this resting cell can be related to the narcotic concentration by an expression derived from the law of mass action.
2. To account for the relation between the concentration of the narcotic and its effect on respiration in the fertilized eggs, it is necessary to conclude that in them the narcotic acts on two parallel respiratory systems. The experimental data can be quantitatively predicted (1) if the reaction of the narcotic on the two systems is governed by the law of mass action and (2) if 40 per cent of the oxygen consumption is mediated by one system, the "activity" system, and the remainder by the other, the "resting" or "basal" system.
3. The mass law constants applying to the resting system in the fertilized egg are similar to those for the single system functioning in the unfertilized egg so that these two respiratory systems are probably identical.
4. The concentrations of the narcotics just sufficient to abolish cell division affect primarily the activity system, the existence of which was inferred from the respiratory experiments. It is concluded that normal cell division requires specifically the normal function of the activity system, that in fact the energy for cell division is made available through that system.

We wish to record our appreciation of the assistance given by Mr. G. R.

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