EFFECTS OF FERTILIZATION AND DEVELOPMENT ON THE OXIDATION OF CARBON MONOXIDE BY EGGS OF STRONGYLOCENTROTUS AND URECHIS AS DETERMINED BY USE OF C^{13} [1]

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In a previous publication, Black, Epstein and Tyler (1958) presented evidence, obtained by the use of a C^{13} label, for the oxidation of CO in fertilized eggs of the gephyrean worm, *Urechis caupo*. In the light the oxidation of CO is superimposed on the ordinary respiration, so that manometrically an excess gas-uptake is observed. The eggs also oxidize CO in the dark, and this masks, in manometric experiments, an inhibition of respiration by this compound.

Studies by other workers showing excess respiration in the presence of CO, as well as oxidation of this compound, were previously discussed (Black *et al.*, 1958; Rothschild and Tyler, 1958). Pertinent experiments, showing such excess gas-uptake in the presence of CO, have been performed on eggs of the sea urchin (Runnström, 1930; Lindahl, 1939; Rothschild, 1949) and the ascidian (Minganti, 1957), on diapausing grasshopper and silkworm embryos (Bodine and Boell, 1934; Wolsky, 1941; Kurland and Schneiderman, 1959), on skeletal and heart muscle of the frog and rat (Fenn and Cobb, 1932a, 1932b; Schmitt and Scott, 1934; Clark, Stannard and Fenn, 1950), on Earle's L-strain cells from the mouse and MK II cells from monkey kidney cultured *in vitro* (Dales and Fisher, 1959), and on leaf tissue of the wild plum (Daly, 1954).

In the early studies on muscle tissue of the frog and rat noted above, the evidence for the oxidation of CO was obtained by manometric methods alone. Similar evidence has been presented by Dales and Fisher for mammalian cells cultured *in vitro*, and by Allen and Root (1957) for whole blood of rats, dogs and men. The use of isotopically labelled CO has provided direct proof of its oxidation in vertebrate muscle tissues (Clark, Stannard and Fenn, 1950) and in whole turtles and mice (Clark *et al.*, 1949). The use of C^{13}-labelled CO also enabled Black *et al.* (1958) to obtain the first direct evidence for its oxidation in marine eggs.

With regard to previous reports of respiratory stimulation by CO in marine eggs, several points of interest may be mentioned. In the sea urchin Lindahl (1939) found a stimulation of respiration of unfertilized eggs by CO both in the light and in the dark. During development, the excess respiration in the light showed a decrease relative to the total respiration. In the dark, the respiration became progressively more inhibited in the presence of CO as development proceeded. A

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similar finding was reported by Minganti (1957) for the eggs of *Phallusia*. These investigators, as well as Runnström (1956), have rejected the possibility of the oxidation of CO in these materials, while Rothschild (1949) considered this to be the most likely mechanism for the excess respiration in the sea urchin.

It was of interest, then, to determine, by use of the C^{13} label, whether or not sea urchin eggs also possess the capacity to oxidize CO and to follow the changes in this property during development in both the sea urchin and *Urechis*.

If one interprets the data of Lindahl (1939) and Minganti (1957) on the premise that CO-oxidation accounts for the excess gas uptake in CO-O_2 mixtures in the light, then their results indicate that during development this capacity decreases relative to the total respiration. The present results with C^{13}-labelled CO show such relative decrease in capacity for CO-oxidation. These experiments also provide data concerning the effect of illumination on the rates of CO-oxidation at various stages in development, and this pertains to the interpretation of CO-inhibition experiments in general.

Material and Methods

Eggs and embryos of the gephyrean worm *Urechis caupo* and the sea urchin *Strongylocentrotus purpuratus* were employed in the experiments. Non-swimming stages were washed by settling in CO_2-free sea water buffered at pH 8.0 with glycylglycine. Top-swimming embryos were collected by filtration through a coarse sintered-glass filter and washed by low-speed centrifugation.

Gas-uptake and CO_2-production were measured with Warburg-Barcroft manometers. In most experiments the vessels employed were of the type designed by Stanley and Tracewell (1955), with two side-arms in the form of hollow stopcocks, which could be closed off from the main chamber, as well as a conventional vented side-arm. In most experiments the vessels contained 3 ml. of egg suspension, 0.3 ml. of 1 N NaOH ("CO_2-free") in the conventional side-arm, 0.4 ml. of 85% phosphoric acid in one stopcock side-arm which was open during the experiment, and 0.3 ml. of "CO_2-free" alkali in the other stopcock side-arm which was closed during the respiration measurement. At the end of an experiment the acid and alkali from the open side-arms were tipped into the main chamber, in order to obtain a measure of the total CO_2 present. After the measurement had been obtained, the closed side-arm containing alkali was opened, allowing the CO_2 to be taken up in the alkali for subsequent analysis of the C^{13}-to-C^{12} ratio. In some experiments conventional vessels were employed, and acid only was tipped in at the end of the run. The alkali was then transferred quantitatively to the Stanley-Tracewell vessels for measurement and recovery of CO_2. In all the instances, retained CO_2 was measured at the beginning of the experiment by tipping acid and alkali into the main chamber of appropriate vessels at the time of the first manometer reading.

The labelled carbon monoxide was prepared from barium carbonate containing 3.85% C^{13}. This was obtained from the Stable Isotopes Division of the Oak Ridge National Laboratories. The method employed was a modification of that described by Bernstein and Taylor (1947), as outlined in a previous paper (Black *et al.*, 1958). In this method the CO_2 generated from the $BaCO_3$ is passed over a zinc dust-asbestos fiber mixture heated to 520° C. in a combustion furnace. The CO

formed is mixed with unlabelled CO and stored over 0.1 N NaOH in a gas-bulb attached to a leveling bottle. After storage over alkali for several days, samples of the CO were taken from the vessel and re-oxidized by passing over copper oxide at 400° C. The CO_2 produced was collected in a small volume of alkali and the C^{13}-to-C^{12} ratio was determined. In all cases the expected ratio was obtained, indicating practically complete conversion of the original labelled CO_2 to CO. Duplicate samples taken two weeks apart from one batch of CO indicated that there was no loss of the CO upon storage over the dilute alkali.

After attachment of the Warburg vessels to their manometers the experimental vessels (two in most experiments) were connected to a manifold so that they could be gassed with oxygen simultaneously. Each vessel was flushed with one liter of CO_2-free oxygen from a measuring bottle. The two vessels were then evacuated simultaneously by means of a Toepler pump to ⅕ atmosphere, and refilled with the labelled CO, as previously described. These procedures required about 30 minutes, and 10 minutes were allowed for equilibration of the vessels in the water bath. Control vessels were left open to the air or gassed with 80% CO–20% O_2 during this time. The experiments were run at 19° C. Shaker speed was 95 c.p.m. at 3 cm. stroke. Illumination was provided by a bank of 30-watt reflector-type G.E. incandescent lamps located below a glass shelf of the water bath. The intensity of the light at the level of the egg suspensions was 1100–1200 foot-candles. For measurement of respiration and CO-oxidation in the dark, one experimental vessel was placed in a lined black bag.

Determination of the C^{13}–C^{12} ratios of the respired CO_2 were made with a Nier mass-spectrometer (Nier, 1947), modified for the detection of small differences in C^{13}–C^{12} ratio (McKinney *et al.*, 1950).[3] In the experiments with eggs of the sea urchins, the CO_2 which was contained in the alkali of the Stanley-Tracewell vessel was diluted with appropriate amounts of a $NaHCO_3$ solution (usually 0.3 ml. of 0.24 *M*) in order to lower the C^{13}–C^{12} ratio to a range that would be most effectively handled with least danger of "contaminating" the mass-spectrometer.

The alkali and bicarbonate were transferred under CO_2-free atmosphere to reaction vessels wherein the CO_2 was liberated for analysis in the mass-spectrometer. Data obtained in the mass-spectrometer were expressed as deviation of the C^{13}–C^{12} ratio of the sample from that of the standard in parts per mil., *i.e.*,

$$\frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000.$$

The values obtained in this manner were used in isotope dilution equations for the determination of the amounts of CO_2 which had been derived from the C^{13}-labelled CO.

Results

Manometric data

The rates of gas-uptake and of CO-oxidation in the light and in the dark are presented in Table I for the developmental stages of *Urechis* and in Table II for those of *Strongylocentrotus*.

[3] The authors are indebted to Mr. Joop Goris for analyzing some of the samples, and to the Department of Geochemistry for the use of the mass-spectrometer.

TABLE I

Rates of respiration and of CO-oxidation in 80% CO/O_2 in light and dark by eggs of Urechis caupo at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	1–8	94	150	125	+56	+31	43.2	21.4
2	20–25	663	839	476	+176	−187	105.6	11.7
3	24–30	724	789	395	+65	−329	89.9	4.6
4	24–30	551	593	327	+42	−224	75.8	5.8
5	52–55.5	853	872	384	+19	−469	65.0	6.6
6	52–55.5	792	815	358	+23	−434	60.6	4.8
7	54–57.5	847	883	384	+36	−463	57.5	4.1

In both species, at all stages studied, the measurements show an increase in rate of gas-uptake of the eggs or embryos in 80% CO/O_2 in the light over those in air. In *Urechis* the amount of excess rate of gas-uptake declines somewhat during development, while in *Strongylocentrotus* it increases slightly. However, in both species,

TABLE II

Rates of respiration and of CO-oxidation in light and dark by eggs of Strongylocentrotus purpuratus, unfertilized and at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	0 (unfertilized)	16.0	29.9	28.5	+13.5	+12.3	8.9	8.0
2	0 (unfertilized)	22.5	40.9	37.5	+18.4	+15.0	13.4	10.6
3	1–5	69.9	97.4	88.3	+27.5	+18.4	20.5	9.9
4	1–7	81.9	118.4	105.0	+36.5	+23.1	24.1	10.8
5	26–30	166.0	190.0	132.0	+24.0	−34.0	25.0	3.4
6	27.5–31.5	200	238	203	+38.0	+3.0	25.0	9.8
7	46–50	211	242	164	+31.0	−47.0	24.4	3.3
8	49–53	264	321	216	+57.0	−46.0	34.0	6.4

the relative excess gas-uptake with respect to the respiration in air is considerably lower in the later embryonic stages than in the unfertilized or freshly fertilized eggs. Thus for *Urechis* the average values of the percentage excess gas-uptake, in 80% CO/O_2 in the light, relative to the respiration in air, are as follows:

	Stage		
	Freshly fert. (1–8 hrs.)	1 day (20–30 hrs.)	2 day (52–57.5 hrs.)
% excess gas-uptake (light)	60	14	3

For *Strongylocentrotus* the average values are:

	Stage			
	Unfertilized	Freshly fert. (1–7 hrs.)	1 day (26–31.5 hrs.)	2-day (46–53 hrs.)
% excess gas-uptake (light)	83	43	17	18

In the dark the eggs of both species show greater gas-uptake in the CO-O_2 atmosphere than in air for the early stages of development and considerable inhibition at the later stages. For *Urechis* the average values of the percentage excess gas-uptake in 80% CO/O_2 in the dark relative to the respiration in air are:

	Stage		
	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+33	−39	−55

For *Strongylocentrotus* the average values are:

	Stage			
	Unfert.	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+71	+28	−10	−20

These results accord with those of Lindahl (1939) on the sea urchin showing, in CO in the dark, excess gas-uptake for the unfertilized and freshly fertilized eggs,

TABLE III

Manometrically measured respiratory quotients of eggs and embryos of Urechis and Strongylocentrotus in air and in CO

Experiment	R.Q. in air	Apparent R.Q. in 80% CO/O_2 in the light	Apparent R.Q. in 80% CO/O_2 in the dark
Strongylocentrotus			
1	0.58	0.63	0.73
2	0.85	0.65	0.77
3	0.81	0.77	0.83
4	0.77	0.71	0.85
5	0.85	0.85	0.92
6	0.76	0.77	0.81
7	0.84	0.91	1.0
8	0.81	0.81	1.04
Urechis			
1	0.96	0.98	0.99
2	0.86	1.05	1.0
3	0.82	1.0	1.0
4	0.81	0.92	0.99
5	1.02	0.96	1.08
6	0.81	0.82	1.00
7	0.91	0.92	1.10

and inhibition of respiration for the later embryos. Also, the results obtained in CO in the light accord with those of Lindahl (1939) on sea urchin eggs and Minganti (1957) on ascidian eggs, which showed that the relative excess gas-uptake decreased progressively as development proceeded.

Determinations of CO_2-production were made, as indicated under *Methods,* in these same experiments. Division of these values by the corresponding total gas-uptake gives the respiratory quotients (R.Q.) and apparent respiratory quotients (A.R.Q.) listed in Table III. The A.R.Q. refers to the experiments done in presence of CO since the occurrence of CO-oxidation means that some of the gas-uptake represents CO. Such oxidation has an R.Q. of 2, but manometrically it would be recorded as an R.Q. of 0.67. One would therefore expect that the values for the A.R.Q. in the vessels run in presence of CO would be lower than the R.Q. values in air wherever CO-oxidation occurred.

TABLE IV

Ratio of dark-to-light CO-oxidation

	Rate of CO-oxidation in the dark divided by the rate in the light	
	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0.79
		0.79
Fertilized egg	0.50	0.48
		0.45
One-day larvae	0.11	0.39
	0.05	0.13
	0.08	
Two-day larvae	0.10	0.18
	0.08	0.13
	0.07	

The data, however, are mostly in the opposite direction. This would indicate that along with an inhibition of ordinary respiration (in the dark) and its own oxidation, the CO may be inducing a relative stimulation of glycolysis, such as has been reported by Daly (1954) for wild plum, Ducet and Rosenberg (1952) for spinach, Marsh and Goddard (1939) for carrot, Laser (1937) for rat retina and mouse chorion, and Dales and Fisher (1959) for mouse L-strain cells. The data also bear on the question of possible CO_2-assimilation. This is considered in the *Discussion.*

Data from mass spectrometer

The data for the rates of oxidation of CO in the light and dark by the developmental stages investigated are presented in columns 8 and 9 of Tables I and II.

For *Urechis,* the rate in the light is about twice as high in 20–30 hour larvae as in the cleaving eggs. In 50–58 hour larvae, the rate is about one and one-half times that of the fertilized egg.

In the dark, the rate of CO-oxidation in *Urechis* (Table I, column 9) is nearly 4 times greater in cleaving eggs than in the swimming embryos.

For *Strongylocentrotus* the rate of CO-oxidation in the light in fertilized eggs is about twice that of the unfertilized eggs. Following the striking change after fertilization, there is not much increase in CO-oxidation in the light during the remainder of the developmental period investigated. In the dark (column 9) there is no major change in CO-oxidation after fertilization and during later development.

A comparison of CO-oxidation in the dark with that in the light is given in Table IV in terms of the ratio of the two rates at the various developmental stages. In both species this ratio decreases in the later stages. Interpretation of this is offered below on the basis of change in the rate of electron transfer by cytochrome oxidase during development.

For comparison of the rates of CO-oxidation with the excess respiration it should be noted that for each mole of CO_2 derived from CO there would be a manometrically measured gas-uptake of one and one-half moles, according to the equation $CO + \frac{1}{2} O_2 \rightarrow CO_2$. If it is assumed that there is no inhibition of the ordinary respiration by CO in the light, then one would expect the values obtained by mass-spectrometry (column 8 of Tables I and II) for CO-oxidation to equal two thirds of the excess gas-uptake determined manometrically (column 6 of Tables I and II). Such an agreement was found in the previously reported (Black *et al.,* 1958) experiments on freshly fertilized eggs of *Urechis*. It is also found in the present data for *Strongylocentrotus* at all stages and the data for *Urechis* at the early stages. However, for *Urechis* at the 52–57.5 hour stage the excess gas-uptake is considerably lower than expected from the values for CO-oxidation. This evidently means that under the conditions of the experiment, at this stage, there is an inhibition of the ordinary respiration by CO in the light. Possibly this is correlated with the high absolute rate of respiration that the *Urechis* embryos exhibit at this stage. Whether or not increase in illumination would overcome the deficiency in excess gas-uptake (in comparison with CO-oxidation) in *Urechis* at this stage has not as yet been determined.

Discussion

Respiratory inhibition by CO

The mass-spectrometric data for CO-oxidation in the dark permit evaluation of "true" CO-inhibition of ordinary respiration. These values for the inhibition are presented in Table V and are derived simply by subtracting one and one half times the values in column 9 of Tables I and II from the corresponding values in column 5. This is then the respiration in the presence of CO in the dark corrected for CO-oxidation, and its percentage difference from the respiration in air (column 3) gives the inhibition values listed in Table V.

These data show no inhibition of ordinary respiration by CO in the dark for unfertilized and freshly fertilized eggs of *Strongylocentrotus* and for freshly fertilized eggs of *Urechis*. For the later stages of both species there is inhibition of ordinary respiration, the amount of which is, in general, greater the greater the absolute rate of respiration of the air-controls.

A comparison of the data showing lack of inhibition with those of related experiments reported in the literature would be difficult in view of uncertainty, in earlier work, as to the extent to which CO-oxidation occurs in the dark. In any case the lack of inhibitory action of CO in the dark on the early developmental

stages does not necessarily imply the absence of a cytochrome system. As Warburg (1927) pointed out, one would not expect a cell to show much inhibition of respiration in the presence of CO unless it was rapidly oxidizing substrate. Thus Runnström (1930, 1932) and Örström (1932) found that when the respiratory rate of *Paracentrotus* eggs was increased by means of dimethylparaphenylene diamine it could be inhibited by CO. Other evidence for the operation of a cytochrome system in eggs of *Urechis* has been previously presented (Rothschild and Tyler, 1958). A brief review is given there, and a more extended one by Runnström (1956) deals with this system in sea urchins.

TABLE V

"True" respiratory inhibition by CO in the dark in developmental stages of Urechis and Strongylocentrotus, obtained after correcting for CO-oxidation

	Percentage inhibition of respiration by CO in the dark	
Period of development	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0
		0
Fertilized egg	0	−5.0
		−9.0
One-day larvae	34	23.5
	46	6.0
	42	
Two-day larvae	56	24.6
	56	21.8
	55	

Rothschild (1949) reported an inhibitory effect (Av. 38%) of light on the respiration of unfertilized *Psammechinus* eggs and a smaller effect (Av. 9%) on that of the fertilized eggs. This effect was not found with fertilized eggs of *Urechis* (Rothschild and Tyler, 1958), nor with unfertilized or fertilized eggs of *Phallusia* (Minganti, 1957). It has not, as yet, been investigated with eggs of *Strongylocentrotus*. If such effect occurs with this material, the correction for it would be in the direction that would show an inhibitory action of CO on the respiration of the unfertilized or early stage eggs of this species.

Oxidation of CO

The values given for rates of oxidation of CO in Tables I and II involve the assumption that no CO-fixation occurred and that there was no assimilation of the CO_2 derived from CO in any of the developmental stages. Hultin and Wessel (1952) have found that CO_2-fixation occurs in all stages of the development of the sea urchin pluteus. The extent to which metabolic CO_2 was assimilated in our experiments is not known. If assimilation occurs, then the correction for this would result in larger values for CO-oxidation than those presented here.

From the R.Q. data presented it appears that assimilation cannot account for more than a small fraction of the metabolically produced CO_2. If we assume unity as the expected value of the R.Q. in the absence of assimilation and take 0.85 as a representative value obtained in the present experiments, then there could be

15% CO_2 assimilation. The values for CO-oxidation could then be that much greater than listed.

The participation of Warburg's "iron-containing respiratory ferment" in the oxidation of CO was postulated by Fenn and Cobb (1932b). Subsequently, Breckenridge (1953), using C^{14}-labelled CO and both crude and purified preparations of cytochrome oxidase from heart muscle of the pig, showed that this enzyme is indeed responsible for the oxidation of CO, in the presence of cytochrome *c* and hydroquinone. Evidence somewhat similar to that of Breckenridge is presented in an accompanying paper (Black and Tyler, 1959) for the participation of cytochrome oxidase in CO-oxidation by eggs of the sea urchin.

Breckenridge has based his explanation for the oxidation of CO on the hypothesis that three molecules of cytochrome *a* and one of a_3 are combined in a single unit, as proposed by Ball *et al.* (1951). According to Breckenridge, if as many as three iron atoms of a tetraheme unit are oxidized and the fourth is reduced and combined with CO, then the CO can be oxidized. On the other hand, if as many as three of the iron atoms are reduced, combination of a_3 with CO will inhibit cytochrome oxidase activity (and presumably the oxidation of CO). He has cited evidence to show that such an inhibition would be expected when the rate of electron-transfer is high (*i.e.,* when cytochrome *c* and reducing substrate are present in excess).

Breckenridge has indicated that under conditions of rapid electron transfer, one might expect a relatively large fraction of the iron to be reduced as compared with the fraction when electron transfer is slow. In the dark, nearly all the reduced iron of a_3 would combine with CO, whereas in the light, even when electron transfer is rapid, only a small fraction of the a_3-iron would be associated with CO, the proportion depending on the intensity of light. Under these conditions, therefore, in the light, CO-oxidation would be accelerated, up to a point, by increasing rates of electron transfer, whereas in the dark, the oxidation of CO would be inhibited when the electron transfer is rapid. The ratio of dark-to-light oxidation would thus decrease with increasing rates of electron transfer. Such a decrease in ratio is found in the developing eggs of *Urechis* and *Strongylocentrotus* as the respiratory rate increases (Table III). If one accepts the above interpretation, it may tentatively be concluded that the rate of electron transfer by the cytochrome system of unfertilized eggs and of early developmental stages is low; *i.e.,* the cytochrome a-a_3 unit is highly "unsaturated" with reducing substrate. This interpretation is consistent with the lack of respiratory inhibition by CO in these stages in the dark. During later development, the cytochrome oxidase molecules become more nearly "saturated" with reducing substrate, leading to more rapid electron transfer, greater respiratory inhibition by CO in the dark, and a lower rate of oxidation of this compound. This interpretation has been used by other authors to explain the differential effects of CO on the respiration of diapausing and non-diapausing silkworm embryos (Kurland and Schneiderman, 1959) and of developing eggs of the sea urchin (Runnström, 1930).

Summary

1. The rates of gas-uptake and oxidation of CO (labelled with C^{13}) have been determined for developing eggs of the gephyrean, *Urechis caupo,* and for unferti-

lized eggs and developing embryos of the sea urchin, *Strongylocentrotus purpuratus*. In the light in 80% CO/20% O_2, there is excess gas-uptake over that of the air-controls at all stages of development, but the percentage of excess uptake falls off as development proceeds. In the dark there is increasing inhibition of respiration by CO during development.

2. The rate of CO-oxidation in the light increases less than two-fold in *Urechis* during 50 hours of development. In *Strongylocentrotus* the rate of CO-oxidation in the light nearly doubles after fertilization, but increases relatively little during later development.

3. In the dark, in *Urechis*, the rate of CO-oxidation during early cleavage is about half that in the light, but the dark-rate decreases five-fold during the swimming stages. In the sea urchin there is only a slight decrease in rate of CO-oxidation during development. The developmental changes in the ratio of dark-to-light oxidation of CO are interpreted on the basis of an increasing degree of saturation of cytochrome oxidase with its reducing substrate.

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