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THE OXIDATION OF CARBON MONOXIDE BY FERTILIZED EGGS OF URECHIS CAUPO SHOWN BY USE OF A C^{13} LABEL[1]

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In some previous experiments (Rothschild and Tyler, 1958) with eggs of *Urechis*, it was found that the rate of respiration in the presence of carbon monoxide (95% CO: 5% O_2) in the light was greatly increased above that of the controls (95% N_2: 5% O_2). The average increase amounted to 85 per cent. In the dark there was a slight decrease, averaging about 15 per cent.

In many earlier investigations on eggs and other tissues of various animals and plants there have been reports of a stimulating action of CO on respiratory rate. Examples of this are found in experiments on eggs of sea urchins by Runnström (1930), Lindahl (1939) and Rothschild (1949); on ascidian eggs by Minganti (1957); on diapausing grasshopper- and silkworm-embryos by Bodine and Boell (1934) and by Wolsky (1941); on skeletal and heart muscle of frog and rat by Fenn and Cobb (1932a, 1932b), Schmitt and Scott (1934), and Clark, Stannard and Fenn (1950); on leaf tissue of the wild plum by Daly (1954).

In the experiments on vertebrate muscle tissues, Fenn and Cobb (1932b) and Clark, Stannard and Fenn (1950) obtained evidence that CO is oxidized to CO_2. Clark *et al.* (1949) also reported that intact whole turtles and mice could effect such oxidation of CO when this was administered at very low tensions. In the experiments on plum-leaves, on the other hand, Daly (1954) found that the increased gas-uptake in the presence of CO represents a stimulation of ordinary respiration rather than an oxidation of the CO. The question of whether or not the stimulation of respiration in eggs of sea urchins and ascidians is due to oxidation of the CO was considered by Lindahl (1939), Minganti (1957) and Rothschild (1949). The former two investigators rejected this view while the latter considered it to be the most probable explanation of the increased respiration. In a review of various experiments Runnström (1956) concludes that the evidence is against the possibility of oxidation of CO by sea urchin eggs. However, there has as yet been no direct test of this proposition, such as would be provided by the use of isotopically labelled CO.

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In the present experiments C^{13}-labelled CO was employed in an investigation of the possibility of its oxidation by eggs of *Urechis*. The results show that such oxidation occurs and that it accounts for all of the extra gas-uptake of the eggs in the light. The data also show that an oxidation of CO occurs in darkness, but at a lower rate.

Material and Methods

Eggs of the gephyrean worm *Urechis caupo* were employed in these experiments. They were inseminated in sea water and washed in sea water buffered at pH 8 with 0.01 *M* glycylglycine (Tyler and Horowitz, 1937).

Gas-uptake was measured with Warburg-Barcroft manometers using vessels whose calibration volumes ranged around 25 ml. The vessels generally contained 3 ml. of egg suspension and 0.3 ml. of *M*/1 KOH (low in CO_2). In some experiments in which CO_2 was to be released from the egg suspension as well as from the alkali, magnetically held cups were employed, one for the alkali and one containing 0.3 ml. of 6 *M* H_2SO_4. The contents of these could be separately tipped into the egg suspension at the desired time by removal of an externally supported magnet. The KOH used in the alkali-wells of the manometer vessels was prepared from a saturated solution, in which K_2CO_3 is largely insoluble, and diluted with CO_2-free double-distilled water under CO_2-free air. An analysis of the alkali prepared in this manner gave 0.8×10^{-6} mole of total carbonate per 0.3 ml. In filling the manometer vessels the alkali was introduced last.

The use of C^{13} offers some advantages over C^{14} for these experiments. Use of C^{14} would involve precipitating and weighing very small quantities (less than 4 mg. as $BaCO_3$) of the carbonate derived from the respired CO_2 in the alkali well of the usual manometer vessels. This is unnecessary for the mass spectrometric measurement of C^{13} which provides the required quantitative data in the form of the ratio of C^{13} to C^{12} in the sample. It also avoids such uncertainties as are entailed by the self-absorption of radiation in the measurement of C^{14}. In addition, use of C^{13} eliminates possible health-hazards and possible effects of radiation on the system under investigation.

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 essentially similar to the continuous flow technique described by Bernstein and Taylor (1947). The apparatus consisted of a CO_2 generator connected to a Pyrex combustion tube (8 mm. i.d.), containing about 50 grams of zinc-dust-asbestos fiber (95 : 5), within a combustion-furnace of 18 cm. length, and leading through a three-way stopcock to the top of a storage bulb. The latter was provided also with a bottom stopcock leading to a levelling bottle containing N/10 NaOH. At the start of the preparation the storage bulb was filled with the alkali up to the three-way stopcock. A weighed amount of the C^{13}-enriched $BaCO_3$ was placed in the generator, and the generator and combustion tube, up to the three-way stopcock, were flushed with unlabelled CO. The furnace was set at 520° C. Hydrochloric acid was introduced into the generator at a rate producing about 25 to 50 cc. of CO_2 per minute. Measurements of the volume of fluid displaced in the storage bulb showed

that the amount of CO obtained in this system was close to that expected. After CO_2 generation had stopped, the gas remaining in the generator and combustion tube was flushed into the storage bulb with enough unlabelled CO to make a final volume of one liter. The relative volumes of labelled and unlabelled CO were 362 to 638 for the preparation, giving a C^{13} content of 2.14%. Relative to a C^{13} content of 1.17% found for the CO_2 from *Urechis* eggs respiring in air, this gives 82.9% for the atom percentage excess C^{13} of the preparation. The labelled CO was stored over alkali for at least one day prior to use. Storage over alkali for several weeks showed no change in gas volume, indicating no significant contamination by acidic gases.

After attachment of the Warburg vessels to their manometers they were flushed with one liter or more of oxygen. They were then attached to a Toepler pump and evacuated to one-fifth of the original pressure, precautions being taken, by stoppering the open end of the manometers and closing-off the bottom rubber well with a clamp, to avoid drawing the Brodie's fluid out of the manometers. The C^{13}-labelled CO was then introduced through the three-way stopcock at the top of the manometers, after a preliminary flushing of connecting tubes. By this procedure the CO–O_2 ratio could be fixed with considerable accuracy to the desired value, which was 4 : 1 in the present experiments. About 15 minutes were required for these procedures and 10 minutes were allowed for equilibration in the temperature bath. The control vessels were left open to air during the gassing of the experimental vessels. The experiments were run at 20° 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. This supplied 1100 to 1200 foot-candles at the level of the egg suspensions in the Warburg vessels.

The C^{13} determinations were made with a Nier mass spectrometer (Nier, 1947) modified for detection of relatively small enrichments by McKinney *at al.* (1950). The sensitivity of the instrument is such that differences of two parts in ten thousand in the C^{13}-to-C^{12} ratios can be readily detected. For introduction of the respired CO_2 into the mass spectrometer, the procedure followed in two of the experiments (No. 1 and No. 2) was to transfer the alkali from the center well of the Warburg vessel quantitatively, with CO_2-free water and with precautions to avoid contamination with atmospheric CO_2, to a reaction vessel wherein the CO_2 could be liberated by tipping-in concentrated H_3PO_4 from a side-arm (McCrea, 1950). This was attached to the vacuum-line of the mass spectrometer. In one of the experiments (No. 3), the CO_2 was liberated within the Warburg vessels by tipping acid from one of the contained insert-wells into the egg suspension and the alkali. After measurement of their amounts the CO_2 samples were transferred to the reaction vessels by means of the Toepler pump. In two of the experiments (No. 2 and No. 3) a measured amount of $NaHCO_3$ was added to the reaction vessel in order to decrease the C^{13} enrichment to values within the range best suited for the mass spectrometer. The measurements are corrected for the dilution factor.

Results

Effect of CO on gas-uptake of eggs of Urechis

The relevant respiration-data for three experiments are presented in Table I. The first two are for eggs run in the light starting shortly after fertilization, and the third is a dark-experiment with eggs at a similar period of development.

The increase in gas-uptake reported by Rothschild and Tyler (1958) for freshly fertilized eggs of *Urechis* in the light in 95% CO/O_2 is shown also in the present experiments (No. 1 and No. 2) with 80% CO/O_2. Likewise, the lack of appreciable inhibition in the dark is shown in the results of experiment No. 3. Examination of the eggs at the end of the respiration runs in experiments No. 1 and No. 2 showed no significant difference in rate of development between those in 80% CO/O_2 and those in air. The eggs from experiment No. 3 were not available for examination because of the acidification, but separate experiments on eggs run in the dark in $CO–O_2$ mixtures show only a small amount of inhibition of development, as reported previously (Rothschild and Tyler, 1958).

The data in Table I present amounts of gas-uptake calculated as if the total gas were oxygen. Part of the gas-uptake of the eggs in the $CO–O_2$ mixture could (and, as later shown, does) represent disappearance of CO. However, calculations using the solubility of CO instead of O_2 in the usual formula for converting the manometric pressure difference into volume of gas would change these figures by only 0.1%, since the solubility coefficients of the two gases are of the same order of magnitude and this factor contributes relatively little to the vessel constant. This difference is negligible here.

Experiments No. 1 and No. 2 give values of 154 and 130 mm^3., respectively, for the excess gas uptake. Assuming that this is due to the oxidation of CO ($2\ CO + O_2 \rightarrow 2\ CO_2$), then $\frac{2}{3}$ of these quantities represent the amounts of CO oxidized and the corresponding amounts of CO_2 produced therefrom; namely, 102 and 87 mm^3., respectively. The corresponding control vessels yield 318 and 305 mm^3. of CO_2, respectively, on the basis of an R.Q. of unity (Horowitz, 1940). The percentage of the CO_2 derived from oxidation of CO would therefore be 24.3 for experiment No. 1 and 23.4 for experiment No. 2. These are entered in the last column of Table II as expected values, and involve also the assumption that in the light there is no inhibition of the ordinary respiration.

Table I

Respiration-data for eggs of Urechis used in C^{13}-labelled CO experiments

(1)	(2)	(3)	(4)	(5)	(6)
Experiment	Number of eggs per flask	Time interval of experiment in hours after fertilization	Total gas-uptake		Excess gas-uptake in 80% CO/O_2 ($mm.^3$)
			In air ($mm.^3$)	In 80% CO/O_2 ($mm.^3$)	
1 (light)	389,000	$1\frac{1}{2}$–$8\frac{1}{2}$	318	472	+154
2 (light)	622,000	$1\frac{1}{2}$–$6\frac{1}{2}$	305	435	+130
3 (dark)	421,000	1–10	408	394	−14

TABLE II

Percentage of respired CO_2 derived from oxidation of CO, as determined from measurements of C^{13} in mass spectrometer and as calculated on the assumption that such oxidation accounts for all excess gas-uptake in CO-O_2 mixtures in the light

(1)	(2)	(3)	(4)	(5)	(6)	(7)
		Mass spectrometer data				
Experiment No.	Atom % excess C^{13} in CO used in gas space of manometer vessels	Atom % excess C^{13} in respired CO_2			Percentage of CO_2 derived from oxidation of CO	Expected percentage of total CO_2 derived from CO as calculated from excess gas-uptake in light
		With reference to standard CO_2		With reference to control		
		Experimental vessel	Control vessel	Experimental vessel		
1 (light)	82.9	19.92	0.61	19.31	23.3	24.3
2 (light)	82.9	20.28	0	20.38	24.5	23.4
3 (dark)	82.9	16.23	1.35	14.88	18.0	

A calculation of expected CO-oxidation cannot be made in this way for experiment No. 3 which was run in the dark, wherein both inhibition of ordinary respiration and oxidation of the CO might well take place.

Mass spectrometer data relating to oxidation of CO

The results of determinations of C^{13} abundance in the respired CO_2 of the above three experiments are presented in Table II. The atom percentage excess C^{13} in the CO used in these experiments is listed in the second column of the table. These figures also represent the excess that would be expected if all of the respired CO_2 were derived from oxidation of CO. The values obtained from the mass spectrometer measurements for the excess C^{13} in the CO_2 from experimental, relative to that from control vessels, are given in the fifth column of the table. Division of these figures by the corresponding ones of column two gives the percentages (column 6) of the CO_2 derived from CO-oxidation in these three experiments. Comparison with the expected percentages (column 7) calculated from the manometrically determined extra gas-uptake, on the assumption that all of this surplus in the light is derived from CO-oxidation, shows close agreement in experiments No. 1 and No. 2.

This closeness of agreement may, however, be largely fortuitous as the following considerations of further details of the experiments indicate. In experiment No. 1 the control was an aliquot of the same egg suspension respiring in air. The alkali from both experimental and control flasks was transferred quantitatively to the reaction vessels and no carrier $NaHCO_3$ added. The respective percentages of excess C^{13}, relative to the standard used in the instrument, are given in columns 3 and 4 of the table. The air-control shows a small excess of C^{13} relative to the standard source. This simply reflects variation in C^{13}/C^{12} ratios of living and non-living materials from various sources (*cf.* Craig, 1953). Since the carbon of the respired CO_2 of the air-control is all derived from the eggs this indicates

a higher C^{13} content in the eggs than in the standard. In the absence of other information the best method of applying a correction for the control is uncertain, but it seemed most reasonable to us simply to subtract it from the value for the experimental flask. In any case this correction has relatively little effect on the calculations of CO-oxidation.

In experiment No. 2 the respired CO_2 from the air-control vessel was not subjected to C^{13} analysis. Instead, a second type of control was investigated. This consisted of a preparation of lyophilized eggs that was run along with the experimental flask in the 80% labelled CO-20% O_2 atmosphere in the light. This preparation showed a negligible amount of gas-uptake, and was employed to test for possible exchange of carbon atoms between CO_2 and the labelled CO. For this purpose about 300 mm^3. of CO_2 were introduced into the Warburg flask. The analysis of the CO_2 in the alkali of this flask showed no difference in C^{13} content from that of the standard. This indicates that no significant exchange of carbon atoms between the CO and CO_2 occurs in this system.

The determined value for atom percentage excess C^{13} in the CO_2 of the experimental flask of experiment No. 2 was not corrected for any possible contribution from ordinary respiration since the air control in this experiment was not analyzed for C^{13}. A correction of the same order as in experiment No. 1 would lower very little the calculated percentage of CO_2 derived from oxidation of CO (column 6).

The principal source of uncertainty in these two experiments is CO_2-retention in the egg suspensions of the Warburg flasks. As shown in later experiments the egg suspensions may contain considerable amounts of bicarbonate at the beginning of the experiments, despite the normal precautions to keep this at a low value. This unlabelled bicarbonate would presumably form a common pool during the run with bicarbonate derived both from ordinary respiration and from the oxidation of labelled CO. The CO_2 collected in the alkali for analysis would then have been diluted with the unlabelled CO_2 present in the egg suspension at the start of the experiment. Also, some of the labelled CO_2 produced during the experiment would be retained in the suspension at the end of the run. If corrections were made for the above effects, the values calculated in column 6 for CO-oxidation in experiments No. 1 and No. 2 would be higher than those presented. In other words, the value used for atom percentage excess C^{13} to be expected if only CO-oxidation took place would be lower than those listed in column 2. Therefore, the calculated percentages of CO_2 derived from CO-oxidation in these two experiments represent minimum values.

It should be noted that the expected percentages of CO_2 produced from CO by the eggs, as calculated from excess gas-uptake (column 7), also represent minimum values, since they depend on the assumptions that the R.Q. is 1.0, and that there is no inhibition of ordinary respiration by CO in the light. Lindahl (1939) has shown that in 75% CO/O_2 in the light, the eggs of the sea urchin have a lower R.Q. than one would expect, even if one were to account for all the excess gas-uptake as CO oxidation. This could be due to an inhibition of ordinary respiration by CO in the light, which is masked by the utilization of CO. In the present experiments if an R.Q. of 0.67 instead of 1.0 were assumed for the ordinary respiration, as well as the CO-oxidation, then the expected percentages of CO_2 derived from CO-oxidation (column 7) would be 32 and 30 for experiments No. 1 and No. 2, respectively.

In experiment No. 3 the bicarbonate in the egg suspension, as well as that in the alkali well, was collected for analysis of C^{13} content in the mass spectrometer. A control flask of egg suspension, into which acid was tipped at the time of the first reading of the manometers, provided a measure of unlabelled CO_2 originally present. The retained, as well as the respired, CO_2 was determined before transfer to the reaction vessel of the mass spectrometer, as described in Materials and Methods. The total amounts of CO_2 (375 mm^3. in experimental and 384 mm^3. in control flask) were diluted with 0.5 ml. of carrier 0.04 *M* $NaHCO_3$ (480 mm^3. of CO_2). Initial bicarbonate content of the CO/O_2 blank amounted to 160 mm^3. The corresponding dilution factors applied to the mass spectrometer data were therefore $(375 + 480)/(375 - 160)$ and $(384 + 480)/384$ for experimental and control flasks, respectively. The figures entered in columns 3 and 4 of Table II are corrected for the dilution factor.

The value of 18 per cent for the CO_2 derived from CO-oxidation in this experiment is then not subject to uncertainties of retention and can be considered to represent reasonably closely the extent of CO-oxidation occurring in the dark. Since there is about 3% inhibition of gas-uptake (Table I) in this experiment and since 27% (¾ of 18%) of the gas-uptake represents CO-oxidation, then there is 29% inhibition $(100 - 97\ (0.73))$ of the ordinary respiration by the CO in the dark.

Discussion

The results show that eggs of *Urechis* can oxidize carbon monoxide.This occurs both in the light and in the dark. The amount of carbon monoxide that is oxidized in the light can account for all of the excess gas-uptake that occurs in a $CO–O_2$ mixture. In the dark the percentage of CO_2 derived from CO-oxidation is somewhat less than in the light, according to the present data. It should be noted again that the values obtained for oxidation of CO in the light are probably minimal. In other words, there may be a small amount of inhibition of the "ordinary" respiration in the light which is obscured by the oxidation of CO.

It is possible that in the dark CO may be inhibiting, to some extent, its own oxidation. Clark, Stannard and Fenn (1950) found that sodium azide and hydroxylamine completely blocked the oxidation of CO by skeletal muscle, as measured both by manometric and isotope techniques.

Information available from the literature and from the present experiments does not permit identification of the enzymatic system(s) involved in the oxidation of CO. It seems likely that a haem compound is involved because of the known affinity of CO for the Fe^{++} of such substances. Also, it may well go through cytochrome oxidase. However, tests of cytochrome oxidase preparations from *Urechis* and sea urchin eggs (to be reported later) gave no oxidation of CO.

In certain bacteria CO can serve as the sole carbon source (*cf.* van Niel, 1954). Fixation of CO has been demonstrated in barley leaves (Krall and Tolbert, 1957), in which the labelled carbon appears initially in serine and choline. This fixation occurs in both light and dark but the rate is much higher in the light. The possibility of fixation of CO has not, as yet, been examined in animals, but it does seem likely that some of the CO_2 produced by its oxidation would be assimilated.

As previously reported (Rothschild and Tyler, 1958) and as noted here, the development of the eggs was not significantly accelerated or retarded in the $CO–O_2$

mixtures in the light. It might appear, then, that the energy released by the oxidation of the CO is not put to useful developmental work in this system. However, it should be noted that the CO-oxidation would provide much less energy per mole of carbon than the oxidation of the ordinary substrates of the cell. So, even if the energy were utilized, the increase in developmental rate might be too small to be readily detected under the present conditions in which roughly 25 per cent of the respiration is attributed to oxidation of CO. Furthermore, as indicated above, the figure of 25 per cent is a minimum value. Some inhibition of ordinary respiration could be occurring in the light. If, for example, the inhibition amounted to 25 per cent and if it is assumed that oxidation of CO supplies half as much energy per mole of carbon as does the ordinary respiration, then the total rate of energy supply would be the same for eggs in 80% CO/O_2 in the light as for eggs respiring in air. It is then possible that the energy released by oxidation of CO is utilized by the cell for developmental work.

Summary

1. The fertilized eggs of *Urechis caupo* have been found to oxidize CO to CO_2 both in the light and in the dark. This has been shown by the use of C^{13}-labelled CO. In the light there is a previously described increase in gas-uptake in 80% CO/O_2 as compared with air. All of this excess gas-uptake can be attributed to the oxidation of CO.

2. In the dark the percentage of respiratory CO_2 derived from CO is less than in the light. If the oxidation of CO is subtracted from the total gas uptake, the "ordinary" respiration is shown to be inhibited about 29% in the dark by 80% CO/O_2.

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