Comp. Biochem. Physiol., 1966, *Vol.* 18, *pp.* 201 *to* 207. *Pergamon Press Ltd. Printed in Great Britain*

CARBON MONOXIDE CONCENTRATIONS AND THE EFFECT OF AMINOPTERIN ON ITS PRODUCTION IN THE GAS BLADDER OF *PHYSALIA PHYSALIS*

WILLIAM E. HAHN and D. EUGENE COPELAND

Department of Zoology, University of Washington, Seattle, Washington, and Department of Biology, Tulane University, New Orleans, Louisiana, U.S.A.

(*Received* 20 *November* 1965)

Abstract—1. The folic acid inhibitor aminopterin in concentration of 50 μg/ml or more decreases the *in vitro* production of carbon monoxide by *Physalia* gas gland tissue. This supports the possibility that tetrahydrofolates are necessary cofactors in CO production by this coelenterate.

2. CO-producing capacity was highly variable among gas glands tested. CO concentration in the float is also variable. Floats of healthy, average-sized adult *Physalia* were found to contain 8–14 per cent CO by volume. CO concentration in smaller specimens generally ranged from 18–28 per cent.

INTRODUCTION

PHYSALIA PHYSALIS, the Portuguese Man-of-War, metabolically produces carbon monoxide from L-serine (Wittenberg, 1960). The tissue responsible for CO production is the gas gland (pneumadena), which is a specialized area of the thin inner bladder (pneumatosaccus) of the float. Wittenberg *et al.* (1962) have shown that this tissue contains a high concentration of folates. Since the β carbon of L-serine is a known precursor of the N^{10} formyl carbon atom in tetrahydrofolates, Wittenberg suggested that it is probable that tetrahydrofolates carry the one carbon unit from which CO may be derived.

If such is the case, the folic acid inhibitor, aminopterin (Timmis & Felton, 1957), should effect the CO productivity of gas glands incubated *in vitro*. Thus, in the present report, data are presented on the effect of aminopterin on gas glands incubated *in vitro*. Also, values for CO concentration in the floats of animals collected in the open sea are given.

METHODS AND MATERIALS

Animals

Physalia were collected in the Mississippi delta area of the Gulf of Mexico and in the open sea near Port Aransas, Texas. In the Mississippi delta region *Physalia* were most frequently obtained near or at the tidal interface between blue sea water and green to brown Mississippi River water. In the Port Aransas area some specimens were also collected in shallow water (2–4 ft deep) as they were washed toward

shore. Commensal fish, *Nomeus gronovii*, were often associated with *Physalia* collected in the open sea. Animals were used immediately or maintained for a few minutes to an hour in plastic buckets filled with sea water.

Procedures

The gas gland was obtained by dissecting out intact the pneumatosaccus, placing it on bite wax, deflating it and then cutting the circular patch of gas epithelium free with a razor blade. A gas sample was removed for CO analysis just prior to the dissection.

Each gland was divided into two portions. One portion (control) was incubated in a 50 ml syringe containing 15 ml of 0·01 M L-serine (air space of 35 ml) in millipore-filtered sea water adjusted to pH 8·5 with NaOH. The other portion (experimental) was incubated just as the control except aminopterin (10–75 μg per ml) was added to the medium. Incubations were conducted at ambient temperatures, usually at a room temperature of 22°C for a period of 4 hr. Agitation aboard boat was supplied naturally by wave action or in the laboratory by a gyratory shaker timed for a 10 sec shake followed by a 1 min pause.

CO content of the float and that produced by incubated glands was measured colorimetrically by the method of Shepherd (1947). Gas samples of 3–30 ml were diluted in a 1 l. flask and a 25–50 ml sample was then drawn from the flask through a CO indicator tube (Mine Safety Supply Co.). Consistency and accuracy of the CO indicator tubes were checked by testing known dilutions prepared from pure CO.

Gas gland homogenates were prepared in sea water or tris-buffered 3·3% saline by grinding finely minced tissue in a sand mortar followed by brief sonocation. Two ml of homogenate (50 mg gas gland per ml) were incubated in the same manner as the whole tissues.

Float pressure was measured with a calibrated U-tube containing distilled H_2O.

RESULTS

CO *content of individuals*

The concentration of CO in *Physalia* floats previously reported (Wittenberg, 1960; Clark & Lane, 1961; Larimer & Ashby, 1962) showed a marked variation among individuals. *Physalia* used in the former and latter studies were specimens which had been washed ashore. Since *Physalia* quickly degenerate under these conditions, a more accurate range of CO should be obtained if individuals are collected in the open sea and immediately sampled. The results of this select sampling are shown in Table 1. The individuals listed here were generally characterized by having an erect comb, clear blue to greenish color, freedom from a blotchy, opaque appearance of the pneumatocodon and virtual absence of fluid within the float chamber. Animals fitting this description were considered healthy specimens in which variation due to nutritional or physiological state would be minimized. As shown in Table 1, arbitrary division of the specimens into either

an "adult" or small-sized group indicates smaller specimens generally have a higher CO concentration.

A number of gas samples taken at random from non-select specimens collected in the open sea, which included individuals in obviously poor condition, showed a variation in CO concentration from 4·0 to 24·0 per cent, with 70 per cent of the individuals between 8–14 per cent. In comparison, about 50 per cent of the beach-

TABLE 1—CO CONCENTRATION IN THE FLOAT

Specimen	CO concentration per cent
1	11·4
2	10·0
3	15·6
4	12·4
5	11·0
6	15·0
7	8·8
8	5·6
9	9·4
10	8·8
11	11·0
12	14·0
13	11·4
	Large: Average 11·1
14	18·0
15	23·5
16	21·0
17	20·8
18	24·3
19	20·0
20	18·3
	Small: Average 21·7

All specimens listed here were collected in the open sea or surf and specifically selected for a "healthy" appearance. They were analyzed for CO content immediately after collection. Specimens 1–13 had a float length of 4·5–9·5 in. Specimens 14–20 were small animals with a float length of 3 in. or less.

collected specimens contained less than 5 per cent CO. Animals which appear healthy and are collected in the open sea generally have a higher and more consistent float CO concentration.

The pressure inside the float is close to atmospheric pressure. Most individuals measured equaled 0–5 mm of H_2O at 25–27°C. However, when the float is contracting (i.e. when the animal draws the float over on its side) the pressure may rise and fluctuate slightly.

Effect of aminopterin on CO *production*

Table 2 gives the results of CO production obtained from the incubation of gas gland portions in a medium with or without aminopterin. In no case did tissue incubated in the presence of aminopterin produce more CO per unit wet weight

TABLE 2—EFFECT OF AMINOPTERIN ON CO PRODUCTION

Tissue no.	Wt. tissue (mg)	Total μl CO production	μl CO/g/hr
Control			
1	88	350	985
3	70	250	892
5	44	65	360
7	84	95	280
9	58	250	1075
11	104	210	525
13	137	210	382
15	114	200	437
17	85	210	612
19	43	80	465
21	76	320	1052
23	53	110	517
25	38	85	557
27	28	55	492
			$\bar{x} = 616 \pm 75$
Experimental			
2	106	250	590
4	65	190	725
6	26	30	325
8	108	90	210
10	80	250	780
12	94	105	277
14	78	55	172
16	120	190	395
18	105	170	405
20	69	80	390
22	84	200	580
24	60	115	477
26	50	85	425
28	26	40	385
			$\bar{x} = 438 \pm 49$

Experimental gland portions are even numbered and their respective controls (i.e. tissue from the same animal) are the preceding odd numbers listed under "control". For example, No. 1 is the control for No. 2. Experimental medium was identical to the control medium except that 65 μg of aminopterin per ml was added. Incubation time in all cases was 4 hr at room temperature. Weight is wet weight. $\bar{x}$ is $\pm$ one standard error. Difference between $\bar{x}$ of each group using Student's t-test is significant at $P = 0{\cdot}05$.

than respective control tissue taken from the same individual. However, considerable individual variation was encountered, and thus when comparisons are made between individual glands no consistent relationship between gland portions incubated with or without the presence of aminopterin is clear. Considering CO production by each individual gland (control and experimental portions), the inhibitory effect of aminopterin though variable in degree is consistent. Also more active tissue shows a slightly greater degree of inhibition. Taken as a group, there is a wide variation from the mean, and therefore considerable overlap between the control and experimental (aminopterin) incubations exists.

When gas glands taken from animals of questionable physiological condition (i.e. animals washed ashore for an unknown length of time though still alive) were incubated, the amount of CO per unit weight was about half that of fresh specimens, and there was no apparent difference in CO production between control and experimental (aminopterin) incubations. Gas gland homogenates prepared in either sea water or tris-buffered saline were inactive in the production of CO.

DISCUSSION

The gas gland of *Physalis* contains a relatively high (higher than any other animal tissue examined) concentration of folic acid derivatives. Since the gas gland's special function is the production of CO and L-serine has been shown to be an effective *in vitro* substrate, Wittenberg *et al.* (1962) suggested that tetrahydrofolates might be carriers of C_1 units from which CO is evolved. The data of this report support this possibility by indicating a decrease in the CO-producing capacity in a medium containing a relatively high concentration of the folic acid inhibitor, aminopterin. Admittedly, these data are circumstantial since no intermediates have been isolated, but taken together they strongly suggest a tetrahydrofolate-dependent mechanism is involved in CO production by *Physalia.*

Decreased production due to competitive inhibition by aminopterin was not evident except at relatively high concentration (50 μg/ml and above). From limited chromatographic evidence it appears that uptake of aminopterin by whole glands or gland portions is quite small. Therefore, failure to observe noticeable and consistent inhibition at low concentrations (10 μg/ml) during 2–4 hr incubation periods may be due to the limited entry of aminopterin into the cells. For this reason homogenates were prepared in hope that inhibition might be enhanced. However, all homogenates tested were inactive. The reason for this inactivity is not known, but a number of possible explanations may be offered. First, the enzyme system involved in CO production may require some structural arrangement above the molecular level which is destroyed in preparation of the homogenate. Second, since the gas gland is composed of two cell layers, the endodermis (gastrodermis or digestive tissue) and ectodermis, lytic enzymes which are destructive to molecular species involved in CO production may be released from the former during homogenation. It is also possible that sea water or the tris-buffered saline used has some inhibitory action on necessary enzymes freed from the protection of membranes. Larimer (1965) also found homogenates to be inactive in CO production.

Larimer & Ashby (1962) have presented data on CO secretion rate and loss for *Physalia*. They tested CO diffusion rates from the floats and found that the loss of this gas due to diffusion to be approximately 200 μl/hr in the individuals studied. Thus on the basis of their data, maintenance of a pressure differential for CO across the float wall would require the production of 200 μl CO/hr by the gas gland in a "typical" individual. The most active individual Larimer & Ashby tested produced 120·0 μl CO/hr, the lowest 7·5. From data of the present study it can be calculated that 200 mg of active gas gland tissue (200 mg gas glands are commonly found in medium-sized individuals) would be expected to produce about 150 μl CO/hr. From this consideration and the secretion rate data of Larimer & Ashby (1962) it would seem quite unusual for *Physalia* of average size to reinflate a deflated float with apparent ease in a few minutes as stated by Lane (1960).

Variation in CO concentration in the floats of healthy individuals appears to be related somewhat to size. In general, smaller individuals in good condition have higher CO concentrations than larger animals. Since they have a higher surface–volume ratio, small *Physalia* would be expected to lose CO somewhat faster relative to volume than larger individuals. Apparently they produce more gas per unit time relative to float volume and this probably alters the float-atmosphere equilibration. However, limited data of Totton & Mackie (1960) indicate that smaller specimens apparently do not have a proportionally larger gas epithelium relative to float volume. It is not known if the gas diffusion rate for CO through the float layers is the same for large and small individuals. From our study it is clear that the utilization of animals of variable nutritional and physiological state increases markedly the observed range of CO concentration.

Why evolution has favored the production of CO as an inflationary gas is not obvious. Normally this gas is both a respiratory and metabolic poison. However, the low solubility of CO in water (solubility coefficient at 0°C at 760 mm Hg is 0·02142) compared to CO_2 (solubility coefficient 0·759) would seemingly make CO more readily retainable by the thin, moist bladder than a common metabolic gas such as CO_2. Also of significance here is the finding that the concentration of CO normally present in the float of *Physalia* is much below that required for the inhibition of the cytochrome system of this coelenterate under atmospheric oxygen tension (Larimer & Ashby, 1962).

The fact that CO production occurs in a number of algae (Loewus & Delwiche, 1963), during seed germination (Siegel *et al.*, 1962), in the microbe, *Aspergillus flavus* (Westlake *et al.*, 1961), and in green plants (Wilks, 1959) indicates that the biological production of CO is fairly widespread. Whether or not the same general cofactors and enzymes are involved in the biological production of CO in all organisms is not known. *Aspergillus flavus* for example evolves CO in the breakdown of flavonoids. In mammals CO may be formed during the breakdown of hemoglobin (Metz & Sjostrand, 1954). The bathypelagic siphonophore, *Nanomia bijuga*, reportedly contains 90 per cent CO in its numerous minute floats (Pickwell *et al.*, 1964). Because of its high activity this siphonophore might be profitably studied in elucidating the biological production of CO.

SUMMARY

Aminopterin in concentrations of 50 μg/ml or above decreases CO production by *Physalia* gas glands incubated *in vitro*. This is considered to be additional evidence that tetrahydrofolates are involved in the metabolic production of CO in *Physalia*. Lack of effectiveness of lower concentrations may be due to failure of this competitive inhibitor to penetrate cell membranes. Gas glands incubated *in vitro* are most active during the first 4 hr of incubation. Individual variation in activity of glands is very high. Homogenates of the gland did not produce CO when incubated in the same medium used for gland portions. CO concentration in floats of healthy average adult individuals is usually around 8–14 per cent by volume. Small individuals often have a CO concentration noticeably higher than larger specimens (18–28 per cent).

Acknowledgements—We are thankful to Jonathan Wittenberg for consultation on experimental design. The cooperation of Patrick Parker and other members of the staff at the University of Texas Marine Institute is gratefully acknowledged. Support was received from grant GB-676, National Science Foundation.

REFERENCES

CLARK F. E. & LANE C. E. (1961) Composition of float gases of *Physalia physalis*. *Proc. Soc. exp. Biol. Med.* **107**, 673–674.

LANE C. E. (1960) The Portuguese Man-of-War. *Scient. Am.* **202**, 158–168.

LARIMER J. L. (1965) Personal communication.

LARIMER J. L. & ASHBY E. A. (1962) Float gases, gas secretion and tissue respiration in the Portuguese Man-of-War *Physalia*. *J. cell. comp. Physiol.* **60**, 41–47.

LOEWUS M. W. & DELWICHE C. C. (1963) Carbon monoxide production by algae. *Pl. Physiol.* **38**, 371–375.

METZ G. & SJOSTRAND T. (1954) Formation and elimination of CO in mammals. *Acta Physiol. Scand.* **31**, 384.

PICKWELL G. V., BARHAM E. G. & WILTON J. W. (1964) Carbon monoxide production by a bathypelagic siphonophore. *Science, N.Y.* **144**, 860–862.

SHEPHERD M. (1947) Rapid determination of small amounts of carbon monoxide. *Analyt. Chem.* **19**, 77–81.

SIEGEL S. M., RENWICH R. & ROSEN L. A. (1962) Formation of carbon monoxide during seed germination and seedling growth. *Science, N.Y.* **137**, 683–684.

TIMMIS G. M. & FELTON D. G. I. (1957) Structure activity relations in two new series of antifolic acids. *J. Pharm. Pharmac.* **9**, 46–67.

TOTTON A. K. & MACKIE G. O. (1960) Studies on *Physalia physalis* (L.). *"Discovery" Rep.* **30**, 301–408.

WESTLAKE D. W. S., ROXBURGH J. M. & TALBOT C. (1961) Microbial production of carbon monoxide from flavonoids. *Nature, Lond.* **189**, 510–511.

WILKS S. S. (1959) Carbon monoxide in green plants. *Science, N.Y.* **129**, 964–966.

WITTENBERG J. B. (1960) The source of carbon monoxide in the float of the Portuguese Man-of-War, *Physalia physalis* L. *J. exp. Biol.* **37**, 698–705.

WITTENBERG J. B., NORONHA J. M. & SILVERMAN M. (1962) Folic acid derivatives in the gas gland of *Physalia physalis* L. *Biochem. J.* **85**, 9–15.