ALTERATION BY DINITROCRESOL OF PATHWAYS FOR GLUCOSE OXIDATION IN EGGS OF ARBACIA PUNCTULATA

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Some years ago it was observed that dinitrocresol and other substituted phenols could block the cleavage of fertilized sea urchin eggs in a reversible manner (1–3). These agents have a number of effects on energy-yielding processes which are potentially related to those on cleavage: In low concentrations they inhibit aerobic phosphorylation (4–8), but not the phosphorylation associated with glycolysis (9); they also accelerate breakdown of stored high energy phosphate compounds (10); they inhibit glucose-6-phosphate dehydrogenase of yeast (11) and *Arbacia* eggs (12); and, at rather higher concentrations, they inhibit two flavoprotein enzymes, D-amino acid oxidase (13), and cytochrome reductase (11).

The present paper deals with hitherto unrecognized effects of dinitrocresol which may be pertinent both to its general ability to suppress anabolic and assimilative processes (2, 4, 14–16) and to the ability of substituted phenols to stimulate oxygen consumption. Earlier experiments had indicated that *Arbacia* eggs utilize glucose predominantly *via* the so called hexose monophosphate (TPN) shunt, by which the 1-carbon is preferentially converted to CO_2 (12, 17). In studying the oxidation of glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} by *Arbacia* eggs it has now been found[1] that dinitrocresol apparently diverts glucose oxidation from the TPN shunt pathway into another pathway, presumably that *via* glycolysis and the tricarboxylic acid cycle, in which the 6- , 2- , and 1-carbons of glucose are transformed to $C^{14}O_2$ at comparable rates. These observations are of interest in relation to the suggestion that various regulatory factors, including hormones, may act in part by diverting metabolites from one pathway into another (27, 28).

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[1] A preliminary account of this work was presented in abstract form at the General Scientific Meeting of the Marine Biological Laboratory, August, 1955 (29, 30).

Experimental Methods and Materials

Eggs were obtained and handled as previously described (17, 18). Formation of $C^{14}O_2$ and other products was measured over a 2 hour period for eggs at three stages: (*a*) unfertilized; (*b*) beginning 25 minutes after fertilization; (*c*) beginning 24 hours after fertilization and development at 25°C., when the eggs were swimming plutei (19). Incubation was carried out in Warburg vessels at 20°C. The main compartment received 4 ml. of a suspension of 2 per cent eggs (by volume) in sea water or in solutions of dinitrocresol in sea water at pH 8, as shown in the tables; the center well contained 0.5 ml. 0.3 N NaOH, the side arm, 0.5 ml. 0.1 N HCl. The flasks were shaken for 2 hours, after which the HCl was tipped from the side arm into the main compartment; shaking was continued for 60 minutes to displace CO_2 into the center well. The $BaC^{14}O_3$ and other fractions were isolated, and their radioactivity measured, as described elsewhere (17).

The *total* glucose concentration in the sea water of the incubation medium was 0.0006 M in every experiment. Each radioactive sugar was diluted with an appropriate amount of non-labelled glucose so that the contents of one incubation flask, 4 ml. of 2 per cent egg suspension, contained 644,000 C.P.M. at the beginning of incubation. The specific activity of the glucose was 270,000 C.P.M. per micromole under the counting conditions employed.

All results are expressed in terms of the changes occurring in a single incubation flask over a 2 hour period at 20°C. Each flask contained 80 c.mm. of eggs or embryos, having a wet weight of approximately 88 mg. and a dry weight of about 21 mg., of which about 14 mg. was protein (18); the number of eggs per flask was approximately 380,000.

The purchase of the labelled sugars was made from a grant-in-aid by the Atomic Energy Commission to the Marine Biological Laboratory.

RESULTS

Two concentrations of dinitrocresol (DNC) were tested; the one, 8×10^{-6} M, is that which gives optimum stimulation of oxygen consumption in *Arbacia* eggs and reduces the rate of cleavage of eggs and the aerobic phosphorylation in egg extracts by some 50 per cent; the other concentration, 1.28×10^{-4} M, is above that for optimum respiratory stimulation and blocks cleavage and aerobic phosphorylation completely (2, 8).

Glucose oxidation to $C^{14}O_2$ (Table I).—The control values for normal eggs and embryos illustrate the fact, discussed in detail elsewhere (17), that $C^{14}O_2$ production from glucose-1-C^{14} was very much more rapid than from glucose-2-C^{14} or glucose-6-C^{14}. The ratios of $C^{14}O_2$ from glucose-2-C^{14} and from glucose-6-C^{14} to that from glucose-1-C^{14} were, respectively: for unfertilized eggs, 0.32 and 0.06; for eggs fertilized 25 minutes before start of incubation, 0.59 and 0.17; for 24 hour embryos (average of two experiments), 0.47 and 0.33.

Dinitrocresol produced an increase in $C^{14}O_2$ formation from glucose-6-C^{14}; with 8×10^{-6} M DNC the total radioactivity collected as $BaC^{14}O_3$ was raised from 340 to 9,400 C.P.M. for unfertilized eggs, from 570 to 3,200 for eggs fertil-

ized 25 minutes, and from 18,000 to 67,000 for 24 hour embryos (Experiment 21 W, see also 28 W). There was apparently a concomitant inhibition of the original preferential pathway for carbon-1 to $C^{14}O_2$: if it is assumed that the new pathway transforms the 1- carbon to $C^{14}O_2$ at the same rate as the 6-carbon, it may be calculated that in 8×10^{-6} M DNC the residue of the original pathway for unfertilized eggs is 11,300 − 9,400 = 1,900 as compared to the control of 5,900 − 340 = 5,560. Similarly, for the fertilized eggs the respective values

TABLE I

Effect of Dinitrocresol (DNC) on Oxygen Consumption and $C^{14}O_2$ Formation from Glucose-1-C^{14}, Glucose-2-C^{14}, and Glucose-6-C^{14} by Arbacia Eggs and Embryos during 2 Hours of Incubation at 20°C.

Each sample contained 88 mg. eggs or embryos wet weight. For details of technique, see Experimental Methods.

Experiment No.	Date	State of egg development at start of incubation	Concentration DNC	O_2 consumed	Total c.p.m. into $BaCO_3$ from:				
					Glucose-1-C^{14}	Glucose-2-C^{14}	g-2-C^{14} / g-1-C^{14}	Glucose-6-C^{14}	g-6-C^{14} / g-1-C^{14}
	1955		*moles per liter*	*c.mm.*					
20W	7–26	Unfertilized	0	12	5900	1900	0.32	340	0.06
			8×10^{-6}	76	11,300	12,300	1.08	9400	0.83
			1.28×10^{-4}	31	6600	4700	0.71	1600	0.24
19W	7–25	Fertilized 25 min.	0	55	3400	2000	0.59	570	0.17
			8×10^{-6}	139	4800	4400	0.92	3200	0.67
			1.28×10^{-4}	63	4500	4000	0.89	2200	0.49
21W	7–28	24 hr. embryos	0	143	52,000	24,000	0.46	18,000	0.35
			8×10^{-6}	272	83,000	87,000	1.05	67,000	0.81
			1.28×10^{-4}	211	88,000	98,000	1.10	82,000	0.93
28W	8–12	24 hr. embryos	0	119	40,000	19,000	0.47	12,000	0.30
			8×10^{-6}	218	52,000	43,000	0.83	40,000	0.77
			1.28×10^{-4}	178	64,000	68,000	1.06	62,000	0.97

are: residue, 1,600, control, 2,830; for embryos (Experiment 21 W): residue, 16,000, control, 34,000.

The effects with 1.28×10^{-4} M DNC were more complex, but at each stage of development there was an apparent inhibition of the pathway for preferential oxidation of carbon-1 of glucose.

The 8×10^{-6} M dinitrocresol also produced an increase in $C^{14}O_2$ formation from glucose-2-C^{14}; this increase was either not significantly different from, or slightly larger than, the increase in $C^{14}O_2$ from glucose-6-C^{14}. The possible significance of these observations will be discussed below.

The question arose to what extent the DNC-induced increase in $C^{14}O_2$ from

glucose-C^{14} might be due to an increase in utilization of glucose from the medium, especially as DNC has been found to increase glucose utilization by ascites tumor cells (20). Accordingly, measurements of glucose uptake and glycogen breakdown were made. Glucose uptake from the medium by unfertilized eggs was approximately doubled (Table II) by 8×10^{-6} M DNC, while the $C^{14}O_2$ from glucose-6-C^{14} was increased 28 times (Table I); the glucose uptake by the embryos was not increased at all, while $C^{14}O_2$ from glucose-6-C^{14} was increased 3.5 times. Thus the effect of 8×10^{-6} M DNC on $C^{14}O_2$ formation was not attributable solely to change in glucose uptake.

TABLE II

Consumption of Glucose from Medium and from Egg Polysaccharide (Glycogen) during 2 Hours of Incubation at 20°C.

The quantities of eggs and other conditions were the same as in Table I.

Experiment No.	Date	State of egg development at start of incubation	Concentration DNC	Glucose consumed, μg.	
				From medium	From egg polysaccharide
	1955		*moles per liter*		
24W	8–2	Unfertilized	0	68	157
			8×10^{-6}	118	236
			1.28×10^{-4}	100	162
23W	8–1	24 hr. embryos	0	91	235
			8×10^{-6}	77	287
			1.28×10^{-4}	90	235

No glucose was liberated into the medium during incubation of eggs or embryos without added glucose. No lactate was formed either without or with added glucose in the medium.

Incorporation of C^{14} from Labelled Glucose into other Fractions (Table III).— Both concentrations of DNC reduced incorporation of C^{14} from glucose-C^{14} into the acid-insoluble fraction, which contained the nucleoproteins and some of the lipides of the eggs (Table III). This was consistent with the now well known suppression of anabolic effects in a number of types of cells (14–16, 21)

There were inhibitory effects upon transformation of glucose-C^{14} to other products (Table III). These effects were especially clear-cut for the embryos, where transformation of glucose-C^{14} to all products proceeded in the controls at a much greater rate than in the unfertilized and just fertilized eggs.

DISCUSSION

Arbacia eggs contain a number of the enzymes for utilization of glucose *via* the glycolytic pathway and the tricarboxylic acid cycle (12, 22). They also contain at least four of the enzymes, hexokinase (23), glucose-6-phos-

TABLE III

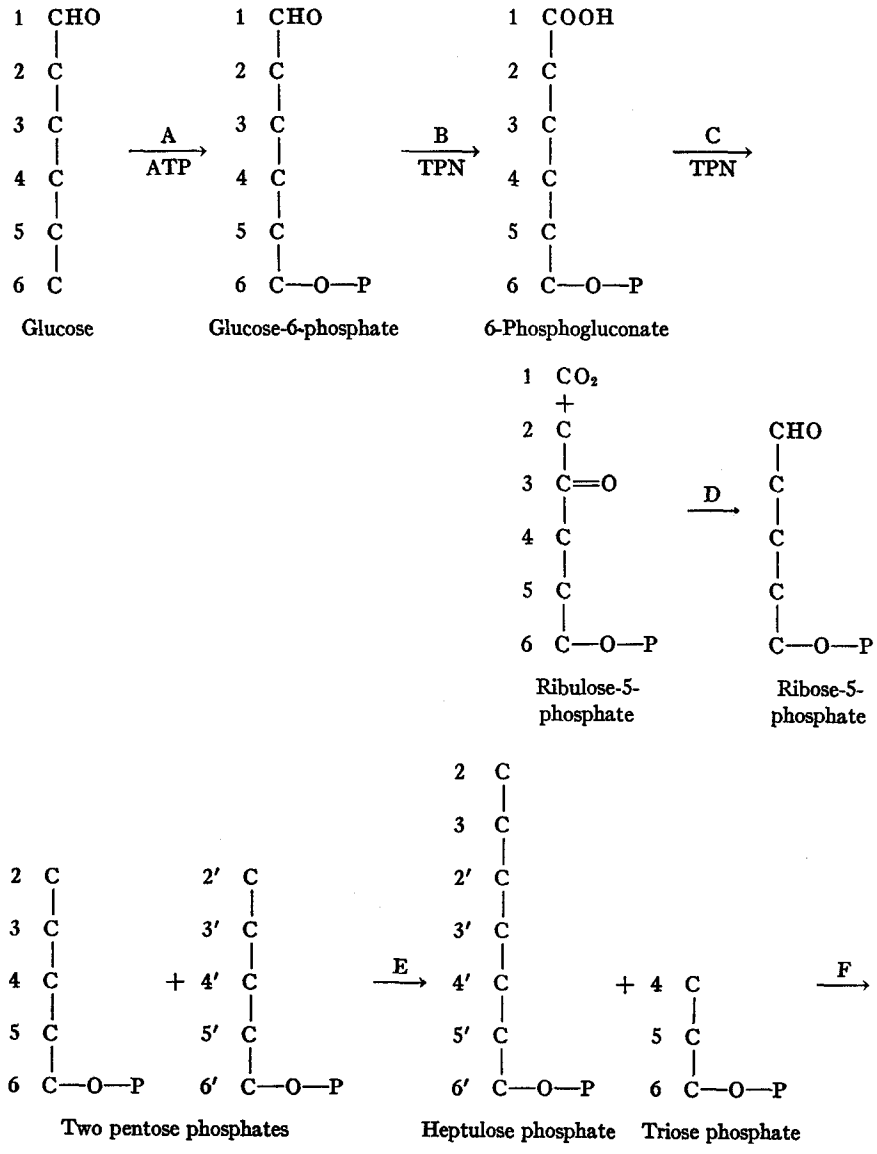
Effects of Dinitrocresol on Incorporation of Radioactivity from Glucose-1-C^{14}, Glucose-2-C^{14}, or Glucose-6-C^{14} into Acid-Insoluble Fraction, Barium Salts Insoluble in Water, and Barium Salts Insoluble in 80 Per Cent Ethanol

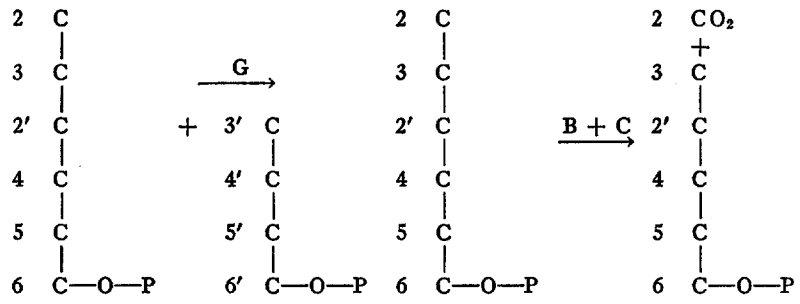
The experiments are the same as those in Table I.

Experiment No.	State of egg development at start of incubation	Concentration DNC	Total C.P.M. from:		
			Glucose-1-C^{14}	Glucose-2-C^{14}	Glucose-6-C^{14}
		moles per liter			
Acid-insoluble fraction					
20W	Unfertilized	0	1300	1700	1400
		8×10^{-6}	300	200	200
		1.28×10^{-4}	60	60	50
19W	Fertilized 25 min.	0	300	300	300
		8×10^{-6}	200	100	200
		1.28×10^{-4}	50	60	50
21W	24 hr. embryos	0	32,000	49,000	42,000
		8×10^{-6}	15,000	18,000	17,000
		1.28×10^{-4}	1600	1400	1600
Barium salts insoluble in water					
20W	Unfertilized	0	4000	5800	5300
		8×10^{-6}	3200	3400	4100
		1.28×10^{-4}	2800	3100	2800
19W	Fertilized 25 min.	0	1300	2300	1900
		8×10^{-6}	900	1200	1100
		1.28×10^{-4}	1200	2100	1600
21W	24 hr. embryos	0	30,000	35,000	35,000
		8×10^{-6}	17,000	24,000	24,000
		1.28×10^{-4}	7900	7700	16,000
Barium salts insoluble in 80 per cent ethanol					
20W	Unfertilized	0	6000	9500	8000
		8×10^{-6}	2400	3800	2800
		1.28×10^{-4}	3800	5300	4600
19W	Fertilized 25 min.	0	4000	8000	7800
		8×10^{-6}	3800	4600	3800
		1.28×10^{-4}	3500	—	8400
21W	24 hr. embryos	0	25,000	40,000	42,000
		8×10^{-6}	7200	16,000	32,000
		1.28×10^{-4}	7200	8600	14,000

phate dehydrogenase (12), 6-phosphogluconate dehydrogenase (12), and hexose phosphate isomerase (23), of the TPN shunt pathway by which the 1-carbon of glucose is transformed preferentially to CO_2 and pentoses are formed. One current version of the shunt pathway is (24):

Pathway for Glucose Oxidation via TPN Shunt





The reactions are: A, hexokinase; B, glucose-6-phosphate dehydrogenase; C, 6-phosphogluconate dehydrogenase; D, pentose phosphate isomerase; E, transketolase; F, transaldolase; G, hexose phosphate isomerase.

According to this pathway the 1-carbon appears as CO_2 when a single glucose molecule has undergone steps A, B, and C; the 2-carbon appears as CO_2 after oxidation of *two glucose molecules* through step G and the repetition of steps B and C; the 6-carbon appears as CO_2 only if the triose phosphate, the tetrose phosphate, or the hexose phosphates reenter the glycolytic cycle at the appropriate points.

From the standpoint of conversion of glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} to $C^{14}O_2$ the important difference between the glycolytic pathway and the TPN shunt pathway is that the former provides two triose phosphate moieties in which the 1- and the 6-carbons enter the pathway toward CO_2 equally rapidly; the TPN shunt pathway, on the other hand, transforms the 1-carbon directly to CO_2 and provides a triose phosphate molecule with no labelling from the 1-position.

It is of interest to consider the results with dinitrocresol in Tables I and III in relation to the known enzymatic events in *Arbacia* eggs and the effects thereon of substituted phenols. The principal finding in Table I is that 8×10^{-6} M dinitrocresol increases $C^{14}O_2$ production from glucose-6-C^{14} relative to that from glucose-1-C^{14}. It is known that substituted phenols inhibit the enzymes for steps B and C in extracts of *Arbacia* eggs (12). This would tend to increase the steady state concentration of glucose-6-phosphate and, in turn the concentration of fructose-6-phosphate and subsequent substrates of the glycolytic cycle. Such a sequence of events favors utilization of glucose-6-phosphate *via* the glycolytic pathway, in which the 1- and 6-carbons appear equally rapidly as CO_2. This may explain, in part at least, the relative increase in $C^{14}O_2$ from carbon-6.

There is another observation in Table I to be accounted for, namely, the finding that the increase in $C^{14}O_2$ from glucose-2-C^{14} produced by 8×10^{-6} M DNC is sometimes larger than the increase in $C^{14}O_2$ from glucose-6-C^{14}.

It may be recalled (25) that the 1- and 6-carbons of glucose do not appear as CO_2 in the tricarboxylic acid cycle until oxalacetate arising from the citrate initially formed reenters the cycle, while the 2-carbon appears as CO_2 when α-ketoglutarate is decarboxylated. Thus, if there were a relatively large pool of four carbon intermediates, arising from amino acid or fatty acid oxidation, to dilute the labelled intermediates from glucose-C^{14}, the $C^{14}O_2$ formation from carbon-2 of glucose would be relatively favored over that from carbon-6, and the observed effects of 8×10^{-6} DNC on relative oxidation of carbons 2 and 6 to $C^{14}O_2$ could be accounted for. There is no direct evidence for such a pool of four carbon intermediates, but there is evidence that amino acids and fatty acids contribute substantially to the total oxidative metabolism of *Arbacia* eggs (18, 26).

The reduction of C^{14} incorporation into the other fractions, which is more or less independent of the position in which the glucose is labelled, may be provisionally interpreted as a consequence of inhibition, by the dinitrocresol, of ATP production *via* aerobic phosphorylation in the eggs (8); the proportionate reductions in synthesis ensuing from the lowered steady state ATP concentration vary according to the concentrations of ATP required to saturate the various synthetic enzymes in question and according to the quantitative energy requirements of the cells at the various stages of development.

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SUMMARY

1. The $C^{14}O_2$ production by *Arbacia* eggs and embryos from glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} has been measured without and with dinitrocresol in the incubation medium. In the absence of the dinitrocresol, the $C^{14}O_2$ production from glucose-1-C^{14} is more rapid than from glucose-2-C^{14} and much more rapid than from glucose-6-C^{14}; this, together with previous findings, indicates that glucose is utilized in *Arbacia* eggs predominantly *via* the TPN shunt rather than *via* the aldolase step of the glycolytic pathway. In the presence of the dinitrocresol, $C^{14}O_2$ from glucose-6-C^{14} approaches that from glucose-1-C^{14}, indicating that, in the presence of this reagent, glucose utilization is diverted from the shunt to the glycolytic pathway.

2. Incorporation of C^{14} from glucose labelled in the 1-, 2-, or 6- positions into other metabolic products of the eggs and embryos is also inhibited by dinitrocresol, particularly incorporation into the acid-insoluble fraction containing nucleoproteins.

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