Effect of Fixation Time on Measurement of 2'-Deoxyribonucleoside 5'-Triphosphate Pools in the Rat Embryo

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Values for the concentrations of deoxyribonucleoside triphosphates in rat embryos on day 12 of gestation, determined by high-pressure liquid chromatography, were artifactually two to three times as high in embryos fixed by cooling in ice/water followed by freezing on solid CO₂, in 20 s, as in those more rapidly fixed in liquid N₂, in 1 s.

It has been shown that rapid fixation of biological samples is necessary to avoid artifactually low values when measuring concentrations of nucleotides and other labile metabolites (Bishop *et al.*, 1960; Mandel & Harth, 1961; Mandel, 1964; Bucher & Swaffield, 1966; Cole *et al.*, 1967; Harrison & Maitra, 1969; Schmidt *et al.*, 1972; Veech *et al.*, 1973). When dealing with bacterial and cell cultures precautions are necessary, but rapid fixation is not difficult, since the dispersed organisms are directly in contact with the fixative solution (e.g., see Walters *et al.*, 1973; Tattersall *et al.*, 1974). By contrast, tissue samples pose a more difficult problem if results representative of true physiological conditions are to be obtained. This is due to anoxia, nutritional deprivation and metabolic changes occurring during the time required to dissect and process the tissue. For example, Mandel & Harth (1961) showed that decapitation of rats directly into liquid N₂, followed by dissection and extraction of the frozen brains, resulted in values for ATP and other nucleoside triphosphate concentrations that were two to four times higher than those obtained for brains first removed during a 20 s ambient-temperature dissection procedure, and then frozen and processed.

Wollenberger *et al.* (1960) used tongs, the jaws of which were constructed from metal blocks, which were first cooled in liquid N₂ and then used to clamp and quick-freeze large tissue pieces and whole organs. This appears to be an effective procedure and has been used to fix samples of regenerating liver before assay for nucleotides (Bucher & Swaffield, 1966) and deoxyribonucleotides (Söderhäll *et al.*, 1973). Veech *et al.* (1973) developed a rapid freeze-blowing technique for fixation of rat brains in which probes are driven into the cranial vault, and air pressure blows the brain tissue into a thin chamber previously cooled with liquid N₂. These are excellent methods, but may not be applicable in all cases, and were not in our studies, where it was desired to exteriorize the rat uterus on day 12 of gestation and rapidly remove the

embryo from its accessory tissues before deoxyribonucleoside triphosphate assay. Schmidt (1976) developed the use of focused-microwave radiation for the fixation of rat brain before choline and acetylcholine assay. Although of general utility, this technique was not satisfactory in our studies, probably because of the high water content and fragility of day-12 rat embryos. Radiation dosage sufficient to fix this tissue caused its general disruption and loss of structural integrity, so that the embryo could not be cleanly separated from surrounding tissues (E. J. Ritter, W. J. Scott & L. M. Bruce, unpublished work).

Two freezing methods for the fixation of day-12 rat embryos were investigated, followed by deoxyribonucleoside triphosphate assay. Of particular interest is the fact that the more rapid method resulted in smaller, rather than larger, values for deoxyribonucleoside triphosphate concentration. This was contrary to expectation, although not unique, e.g. Schmidt *et al.* (1971) observed artifactual increases in cyclic AMP in rat brain after slow fixation. This phenomenon is discussed below in relationship to Quastler's four-factor model (see Cleaver, 1967). We suggest the use of our rapid liquid-N₂ fixation technique where appropriate to the system under study, and stress the importance of rapid fixation of tissue before assay of deoxyribonucleotides and other labile pool components.

Experimental

Sample preparation

Wistar rats were bred and, on day 12 of gestation, anaesthetized and the embryos removed (Ritter *et al.*, 1971). In one dissection procedure the uterus was exteriorized, without interrupting the blood supply, a short slit cut through the antimesometrial uterine wall, and light pressure applied to extrude the embryonic vesicle. This was immediately placed in ice/water, the embryo dissected away from accessory

Table 1. *Deoxyribonucleoside triphosphate concentrations in day-12 rat embryos*

Rat embryos (day 12 of gestation) were fixed by cooling in ice/water and freezing on solid CO₂, during about 20s, or freezing in liquid N₂ during about 1 s, then ground with frozen 0.5M-HClO₄, and assayed for deoxyribonucleoside triphosphate concentrations by h.p.l.c. (high-pressure liquid chromatography). Details of the procedure and assay are described in the Experimental section. Results are means $\pm$ S.E.M.

Method of preparation	No. of samples (litters)	Concentration (nmol/g of tissue wet wt.)			
		dTTP	dCTP	dATP	dGTP
(a) Ice/water-solid CO ₂	11	12.02 $\pm$ 0.87	5.67 $\pm$ 0.40	3.83 $\pm$ 0.32	2.91 $\pm$ 0.20
(b) Liquid N ₂	16	4.25 $\pm$ 0.34	3.13 $\pm$ 0.48	2.58 $\pm$ 0.22	1.11 $\pm$ 0.10
(a) as a percentage of (b)		283	181	148	262

tissues, dried on a spatula with wedges of filter paper, and quickly frozen by touching to the inside surface of a glass vial cooled in solid CO₂. This 'ice/water-solid-CO₂' procedure required an elapsed time of about 20s after extrusion from the uterus. All of the embryos from one litter were pooled as one sample; there were ten to twelve embryos per litter, and individual embryos weighed 40–50 mg. Eleven litters were prepared by this procedure.

In the alternative 'liquid-N₂' procedure the embryonic vesicle was removed as above, but immediately frozen by dropping into liquid N₂, this requiring about 1 s. It was then removed, placed on a block of solid CO₂, and accessory tissues were chipped away. Sixteen litters were prepared by this procedure.

After either procedure the still-frozen pooled embryos were placed in a cold preweighed vial enclosed in a small close-fitting cellular-polystyrene container and quickly weighed, then ground in a solid CO₂-cooled mortar and pestle containing frozen 0.5M-HClO₄. Assay of deoxyribonucleotides was performed by h.p.l.c. as previously described (Ritter & Scott, 1938; Ritter & Bruce, 1979). The above homogenate was thawed and centrifuged (10 min at 1000 g), and the supernatant was neutralized with 4M-KOH to pH 7.5 and treated with acidified NaIO₄. The reaction product was freeze-dried, stored at –20°C and reconstituted with distilled water just before assay. Assay was performed on a model 204 liquid chromatograph (Waters Associates) equipped with two Partisil 10 SAX (Whatman) anion-exchange columns (25 cm $\times$ 4.6 mm internal diam.) in series, maintained at 40°C, followed by a model 400 u.v. detector operated at 254 nm (Waters Associates), an Omni-Scribe recorder (Houston Instruments) and a Chromatopac-E1A integrator (Shimadzu). Elution was carried out with a linear gradient of 0.2M-NH₄H₂PO₄, pH 3.2, starting buffer, to 0.6M-NH₄H₂PO₄, pH 4.4, limiting buffer; the flow rate was 1.0 ml/min for 2 h.

Results

Table 1 presents the values for deoxyribonucleoside triphosphate concentrations (nmol/g) obtained for rat embryos on day 12 of gestation fixed by

the liquid-N₂ procedure, and for those fixed by the ice/water-solid-CO₂ procedure. The latter procedure resulted in values of 283% for dTTP, 181% for dCTP, 148% for dATP and 262% for dGTP, compared with those for the faster liquid-N₂ procedure.

Discussion

The importance of rapid tissue preparation before assay of labile pools cannot be overemphasized, if the values are to indicate those existing under physiological conditions *in vivo*. The measurement of deoxyribonucleotide concentrations is particularly sensitive to fixation artifacts, because of the labile nature and rapid turnover of these DNA precursors (Hauschka, 1973; Krowke *et al.*, 1975).

Since nucleotide studies have shown a decrease in concentrations under conditions of slow fixation, we did not expect the opposite with deoxyribonucleotides. However, all precursor pools should be considered in the light of Quastler's four-factor model (see Cleaver, 1967). That is, constituents enter the pool either through salvage pathways or pathways of synthesis *de novo*, and leave either as components of synthesized molecules, or as metabolic breakdown products. In the present study it is suggested that moderate cooling with ice/water inhibited the incorporation of deoxyribonucleoside triphosphates into DNA and/or their decomposition more quickly than it did their synthesis, leading to a build-up in the deoxyribonucleoside triphosphate pools.

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