RNA and Protein in Nucleolar Structures of Dragonfly Oocytes

Abstract. *Dragonfly and damselfly oocytes regularly contain two nucleolus-like bodies of different sizes, a primary nucleolus and a secondary nucleolus. The primary nucleolus stains more deeply with RNA stains than with an arginine stain; the secondary nucleolus stains conversely. The primary nucleolus incorporates uridine under all conditions tested; the secondary, only after precautions have been taken to preserve soluble RNA. Both incorporate lysine.*

In the typical nucleus, there is only one nucleolus and a single nucleolus organizer region per haploid genome. In large cells with particularly exacting synthetic functions, ribonucleoprotein particles or larger bodies resembling true nucleoli may also occur. Experiments on the incorporation of uridine or cytidine have shown that the synthetic capacities of the various accessory bodies may be very low (*1*) or nonexistent (*2*). In the salivary glands of *Chironomus tentans*, the accessory nucleolus cannot compensate for nucleolar functions in anucleolate embryos, but appears to be hypertrophied in animals heterozygous for absence of the (true) nucleolus organizer region (*2*, discussion). Like puffs, the accessory bodies are often temperature-dependent (*3*, *4*) or their presence is otherwise conditioned. The endobody of the house cricket (*Acheta*) (*2*) and the secondary nucleolus of the dragonfly *Pantala* (*5*) and other anisopterous Odonata (*6*), however, appear to be constant in the cell type in which they occur—diplotene oocytes.

We have investigated the cytochemistry of the primary nucleolus (PN) and the secondary nucleolus (SN) in several species, particularly in 57 female and 5 male larvae of the dragonfly *Cordulia aenea* (Corduliidae). The chromosomal relations of the PN and SN were studied in preparations from final larval instar females. Preparations were stained with Feulgen before squashing and with light green after squashing. Within the PN, there is nucleolar chromatin in the form of Feulgen-positive fibers with six or seven chromomeres. The SN, which is always much smaller than the PN, is intimately associated with a separate bivalent and may be organized by parts of this bivalent. The chromatin is often attached to SN in a cuplike fashion (Fig. 1A).

For staining tests, one of the paired

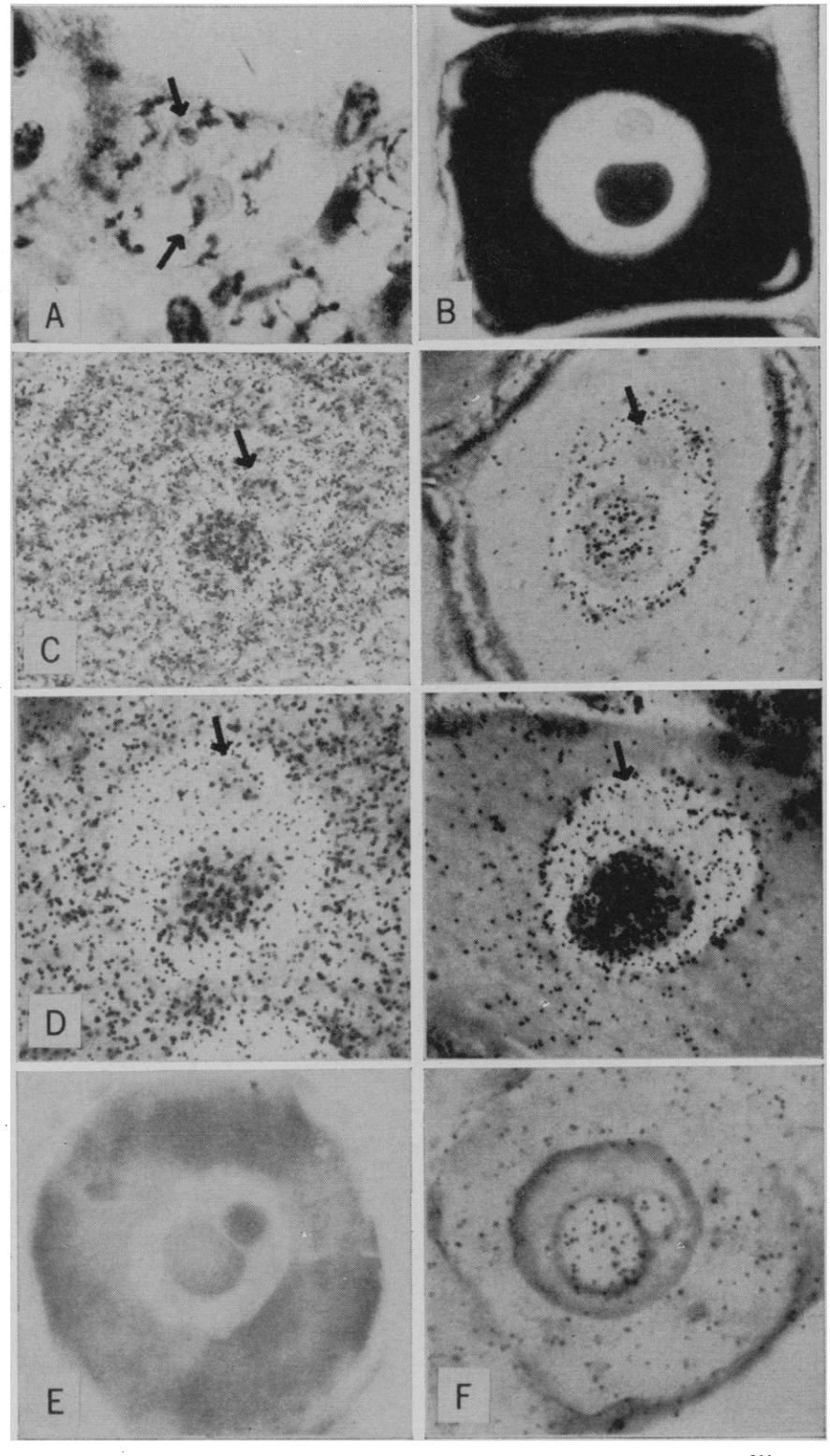

Fig. 1. (A) Feulgen–light green squash of an early diplotene oocyte showing nucleolar chromatin in the PN and association of PN and SN with different bivalents (arrows). (B) Sectioned late diplotene oocyte stained with azure B. (C) Unextracted (left) and extracted (right) preparations of the Woods-Zubay type labeled for 30 minutes with ^{3}H-uridine. Note label in the unextracted and absence of label in the extracted SN (arrows). (D) Similar pair of preparations of the Woods-Zubay type labeled for 3 hours with ^{3}H-uridine. (E) Oocyte stained according to Deitch's modification of the Sakaguchi reaction. (F) Oocyte labeled for 3 hours with ^{3}H-lysine. (× 1000)

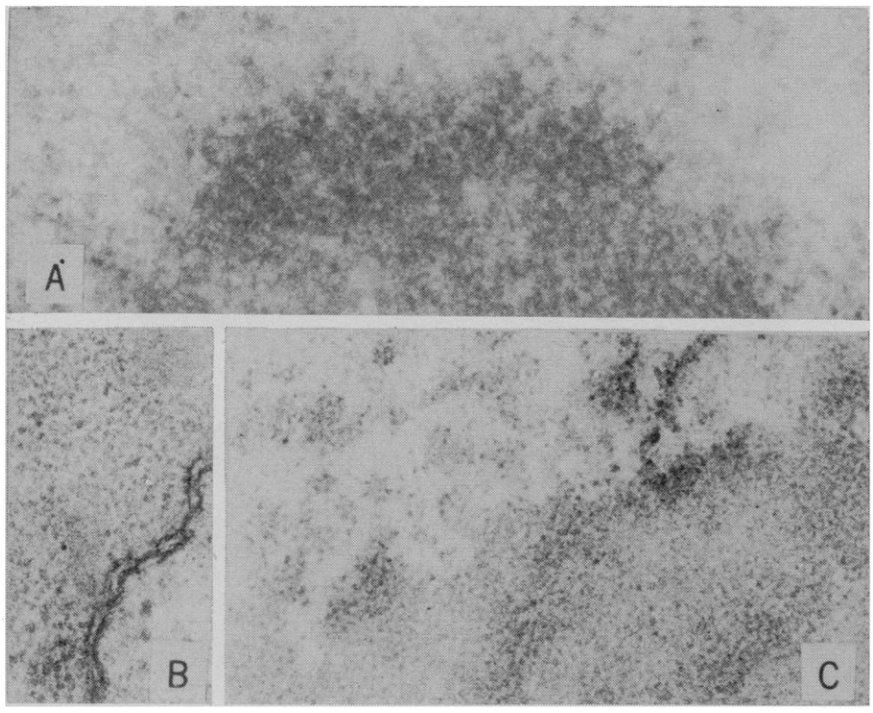

Fig. 2. (A) Marginal area of SN. (B) Cytoplasmic ribosomes in the vicinity of the nuclear membrane. (C) Marginal area of PN. In the PN, note the presence of granular structures of subribosomal size. The sections were stained with uranyl acetate and lead citrate. (× 80,000)

ovaries was fixed in alcohol–acetic acid, the other in neutral 10 percent formalin. The sections were then stained according to one of the following seven methods: Feulgen (*7*) for DNA; cresyl violet (*8*), azure B (*9*), or toluidine blue (*10*) for RNA; Deitch's modification of the Sakaguchi reaction (*11*) for arginine; fast green (*12*) for basic amino acids; and Bloch's fast green–eosin method (*13*) for basic proteins. Ortho- and metachromasy were tested photometrically.

Neither PN nor SN was found to contain sufficient quantities of Feulgen-DNA to be measurable with a microphotometer. With cresyl violet, azure B (Fig. 1B), and toluidine blue, PN stained more intensely than SN. With the two metachromatic stains, azure B and toluidine blue, PN stained bluish and SN reddish violet. With Sakaguchi reaction, SN stained more intensely than PN, that is, the situation was converse of that with the three RNA stains (Fig. 1E). When fast green staining was preceded by extraction of nucleic acids in hot trichloroacetic acid, both PN and SN stained very faintly. The protein components of these two bodies may be partly extractable with nucleic acids. With Bloch's fast green–eosin method, PN stained bright red and SN bluish or reddish grey.

For autoradiography, ovaries of females at the final larval stage were processed in the following way. With a fine glass needle, 25 μl of tritiated uridine [Amersham uridine-T(G), 2300 mc/mole] or lysine (New England Nuclear, 4000 mc/mole) was injected into the abdomen. In preparing the oocytes for ^{3}H-uridine autoradiography, the ovaries were fixed in alcohol–acetic acid or neutral 10 percent formalin (ordinary method) or by freezing-substitution in ethanol (Woods-Zubay method). In experiments with ^{3}H-lysine, only the ordinary preparative method was used. Most of the autoradiograms were stained with Ehrlich's acid haematoxylin through the film (Kodak's AR 10 stripping film).

The periods of incubation in the uridine experiments were 30 minutes, 1, 3, 9, or 30 hours for the ordinary method and 30 minutes, 1 hour, or 3 hours for the Woods-Zubay method. In the lysine experiments, the periods of incubation were 20 minutes, 1 hour, or 3 hours.

In oocytes fixed and treated afterward in the ordinary way, considerable loss of sRNA and other soluble cell components occurred in the alcohol series and at other stages of the procedure. In the Woods-Zubay method, the soluble cell components, particularly sRNA, either were fixed in 67 percent ethanol plus 0.67 percent potassium acetate and left unextracted, or were extracted in a buffer mixture of $MgCl_2$, NaCl, and tris (*14*).

The results obtained in the uridine experiments with the ordinary method display incorporation into nonsoluble RNA's, presumably ribosomal RNA and messenger RNA. In the 30-minute and 1-hour experiments, all the label was confined to the nucleus. With the 3-hour and longer incubations, the cytoplasm also became labeled. The primary nucleolus took up progressively more label with increasing time of incubation, up to 9 hours. The secondary nucleolus was not labeled, except perhaps in material incubated for 30 hours, in which a few grains were usually observed above the SN.

With the Woods-Zubay method, different results were obtained in the extracted and unextracted lines of the procedure. In the extracted line, the labeling pattern in the 30-minute, 1-hour, and 3-hour experiments was the same as with the ordinary method, with strong label in the PN and nucleoplasm, late appearance of label in the cytoplasm, and no label in the SN. In the unextracted line, SN was labeled and the cytoplasm heavily labeled (Fig. 1, C and D). This difference between the extracted and unextracted lines is probably due to the presence of sRNA in the labeling pattern of the unextracted preparations. No relative increase of label in SN was noted with prolonged incubation.

In experiments with ^{3}H-lysine, both PN and SN were found to incorporate this amino acid, although not strongly. Moderate labeling was observed in the nucleus and the cytoplasm (Fig. 1F). The PN took up progressively more label with increasing time of incubation.

In electron micrographs (Philips EM 200), PN and SN look very different in preparations fixed in 3 percent glutaraldehyde, fixed again in 1 percent osmic acid, and stained with both uranyl acetate and lead citrate. The PN has all the components of a true nucleolus, including a fibrillar and a granular part. The SN has a fluffy, fibrous ultrastructure (Fig. 2, A and C).

The PN is obviously the true nucleolus of the dragonfly oocyte—in staining tests, uridine incorporation experiments, and ultrastructural studies it fulfills the criteria defining a nu-

cleolus. Practically all the RNA synthesized or assembled in PN is of the nonsoluble type (in Fig. 1 compare C and D, left and right). We suggest that rRNA is produced in the PN.

The role of SN in cellular metabolism is enigmatic. Our ^{3}H-uridine experiments, performed according to the Woods-Zubay method, indicate that SN is active in synthesis, accumulation, or transit-coupling of soluble RNA. Since SN is rich in arginine and incorporates lysine, it also appears active with regard to proteins containing a good proportion of these amino acids. The SN and PN reach their final size in 30-μ-long oocytes and retain their cytochemical properties in the 300-μ-long oocytes found in freshly moulted adult females.

Our secondary nucleolus, a name originating from the article of Seshachar and Bagga (*5*), is entirely different from the true nucleolus present in the same nucleus. Our cytochemical experiments show that the SN of *Cordulia* is closely but not completely homologous with the "endobody" observed by Bier *et al.* (*2*) in *Acheta* and other Orthoptera. Close homology with the "accessory nucleoli" described by Das and Alfert (*4*) in the marine echiuroid worm *Urechis* also appears likely. We have noted the secondary nucleolus in 11 species of damselflies (Zygoptera) and dragonflies (Anisoptera) belonging to six families. The secondary nucleolus thus seems to be older than the separation of the Odonata into the Zygoptera and Anisoptera. It is possible that the accessory bodies in the Odonata, Orthoptera, and Echiurida perform largely similar functions and result from clustering of important redundant cistrons responsible for products needed in the early periods of embryonic development.

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Methylmalonic Aciduria: Metabolic Block Localization and Vitamin B_{12} Dependency

Abstract. *Methylmalonic aciduria is an inborn error of metabolism characterized by neonatal or infantile ketoacidosis. Leukocytes isolated from the peripheral blood of a 1-year-old child with this disorder converted negligible quantities of propionate-3-C^{14} to carbon dioxide, but oxidized succinate-1,4-C^{14} normally, an indication of a block in the conversion of propionate to succinate. Parenteral administration of vitamin B_{12} resulted in a reduction in methylmalonic acid excretion and an increase in propionate oxidation by leukocytes in vitro. The results suggest a mutation of methylmalonyl-CoA isomerase, a vitamin B_{12} dependent enzyme which converts methylmalonyl-CoA to succinyl-CoA, and provide the first demonstration of vitamin B_{12} "dependency" in man.*

Methylmalonic acid, barely detectable in the urine of healthy humans ($<$ 2 mg/day), is excreted in large amounts by patients with vitamin B_{12} deficiency (*1*). This reversible biochemical disturbance occurs because methylmalonyl coenzyme A (CoA) isomerase, which catalyzes the conversion of methylmalonyl CoA to succinyl CoA, requires a cobamide coenzyme derived from vitamin B_{12} (*2*). Methylmalonic aciduria has also been observed in newborn infants and young children with severe metabolic acidosis who were not vitamin B_{12} deficient (*3*). The methylmalonic aciduria and acidosis observed in these children has been ascribed to an inborn error in the conversion of methylmalonyl CoA to succinyl CoA due to a mutation of methylmalonyl CoA isomerase.

We have described a 1-year-old boy who excreted 800 to 1000 mg of methylmalonic acid per day and also long-chain ketones during periods of keto-acidosis (*4*). Vitamin B_{12} deficiency was clinically excluded. Ingestion of a high protein diet, valine, or isoleucine increased the excretion of methylmalonic acid and long-chain ketones. Since methylmalonyl CoA is a key intermediate in the catabolism of branched-chain amino acids and fatty acids with an odd number of carbon atoms (Fig. 1), these observations were consistent with the proposed block in the catabolism of methylmalonyl CoA.

We now present evidence in vitro for a defect in propionate catabolism and demonstrate that the administration of vitamin B_{12} modifies significantly the biochemical abnormalities observed in vivo and in vitro.

Leukocytes from fasted patients were isolated from heparinized, venous blood (10 to 30 ml) by differential sedimentation in polyvinyl pyrollidone and subsequent hemolysis of erythrocytes (*5*). The leukocytes were suspended in 1 ml of Krebs-bicarbonate buffer (*p*H 7.4) containing 1.65 μmole of propionic acid and 7.7 μc of

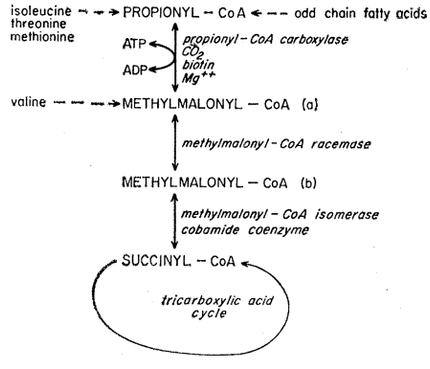

Fig. 1. Pathway of methylmalonic acid formation and catabolism, demonstrating its role as an intermediate in protein and fat metabolism. Broken arrows signify intermediate steps. The cobamide coenzyme active in the isomerization of methylmalonyl CoA to succinyl CoA in mammalian tissue is 5,6-dimethylbenzimidazolyl cobamide 5′-deoxyadenosine (5′-deoxyadenosylcobalamin) (*2*).