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.

LIISA HALKKA
OLLI HALKKA
Department of Genetics, University of Helsinki, Helsinki 10, Finland

References and Notes

1. C. Pelling and W. Beermann, *Nat. Cancer Inst. Monogr.* No. 23 (1966), p. 393.
2. K. Bier, W. Kunz, D. Ribbert, *Chromosoma* **23**, 214 (1967).
3. H. K. Jain, *Heredity* **11**, 23 (1957).
4. N. K. Das and M. Alfert, *Nat. Cancer Inst. Monogr.* No. 23 (1966), p. 337.
5. B. R. Seshachar and S. Bagga, *Growth* **27**, 225 (1963).
6. C. McGill, *Zool. Jahresber.* **23**, 207 (1907); L. T. Hogben, *Proc. Roy. Soc. London Ser. B* **91**, 60 (1921); T. Oksala, *Ann. Acad. Sci. Fenn. Ser. A IV Biol.* **9**, 1 (1945).
7. C. Leuchtenberger, in *General Cytochemical Methods*, J. F. Danielli, Ed. (Academic Press, New York, 1958), vol. 1, pp. 219–278.
8. C. Ritter, H. S. DiStefano, A. Farah, *J. Histochem. Cytochem.* **9**, 97 (1961).
9. M. H. Flax and M. Himes, *Physiol. Zool.* **25**, 295 (1952).
10. C. Pelling, *Chromosoma* **15**, 71 (1964).
11. A. Deitch, *J. Histochem. Cytochem.* **9**, 477 (1961).
12. M. Alfert and J. J. Geschwind, *Proc. Nat. Acad. Sci. U.S.* **39**, 991 (1953).
13. D. P. Bloch, *Chromosoma* **19**, 317 (1966).
14. P. S. Woods and G. Zubay, *Proc. Nat. Acad. Sci. U.S.* **54**, 1705 (1965).

15. We are grateful to V. Varis for help in collecting and identifying specimens, to M. Nyholm for advice in electron microscopy, and to Miss Riitta Lallukka for technical assistance. The investigation was supported by grants from the University of Helsinki and from the National Research Council for Sciences.

20 September 1968

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

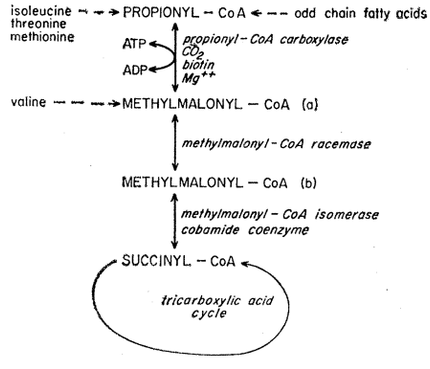

Fig. 1. Pathway of methylmalonic acid formation and catabolism, demonstrating its role as an intermediate in protein and fat metabolism. Broken arrows signify intermediate steps. The cobamide coenzyme active in the isomerization of methylmalonyl CoA to succinyl CoA in mammalian tissue is 5,6-dimethylbenzimidazolyl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

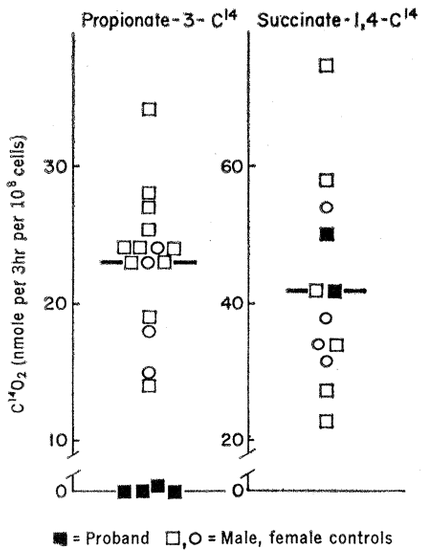

Fig. 2. Formation of $C^{14}O_2$ from propionate-3-C^{14} and succinate-1,4-C^{14} by isolated leukocytes from normal individuals and from a patient with methylmalonic aciduria. The concentration of propionate and succinate in all experiments was 1.65 mmole/liter.

propionate-3-C^{14} or 1.65 μmole of succinic acid and 5.5 μc of succinic acid-1,4-C^{14}. The mixture was gassed with 95 percent oxygen and 5 percent carbon dioxide, sealed, and incubated in modified liquid-scintillation counting vials for 180 minutes at 37°C in a Dubnoff shaker. The reaction was terminated with 1 ml of 6*N* sulfuric acid, and the evolved $C^{14}O_2$ was trapped in hyamine and counted in a liquid-scintillation spectrometer (*6*). Leukocytes from normal subjects oxidized 14 to 30 nmole of labeled propionate to $C^{14}O_2$, but cells from the patient with methylmalonic aciduria produced negligible amounts of $C^{14}O_2$ from propionate in four separate studies over a 3-week interval. The patient's leukocytes oxidized succinate normally, accentuating the significance of the abnormality in propionate oxidation.

These results (Fig. 2) and the observations in vivo (*3, 4*) show that patients with methylmalonic aciduria cannot convert methylmalonyl-CoA to succinyl-CoA, but we are not able to distinguish between an abnormality in the reactions catalyzed by the racemase or the isomerase enzymes (Fig. 1). Additional experiments suggested that methylmalonyl-CoA isomerase is defective in this disease.

On two occasions during a 2-month interval, the patient was hospitalized and placed on a constant diet containing 2 g of protein per kilogram of body weight per day. Urine samples (24-hour collections) were analyzed for methylmalonic acid by a modification of the colorimetric method (*7*) which depends on the diazotization of *p*-nitroaniline yielding a green color in the presence of substituted malonic acids. The patient excreted 800 to 1200 mg of methylmalonic acid per 24 hours (Fig. 3) on this regimen. However, when he was given 1 mg of vitamin B_{12} a day intramuscularly, methylmalonic acid excretion fell to 220 to 280 mg/day, and returned to values before treatment only after the injections were stopped. Coincident with this change in methylmalonic acid excretion was an effect on propionate oxidation by isolated leukocytes (Fig. 3). Whereas $C^{14}O_2$ production from labeled propionate was negligible during control periods, oxidation was observed on four separate occasions while the patient was receiving the vitamin or shortly thereafter.

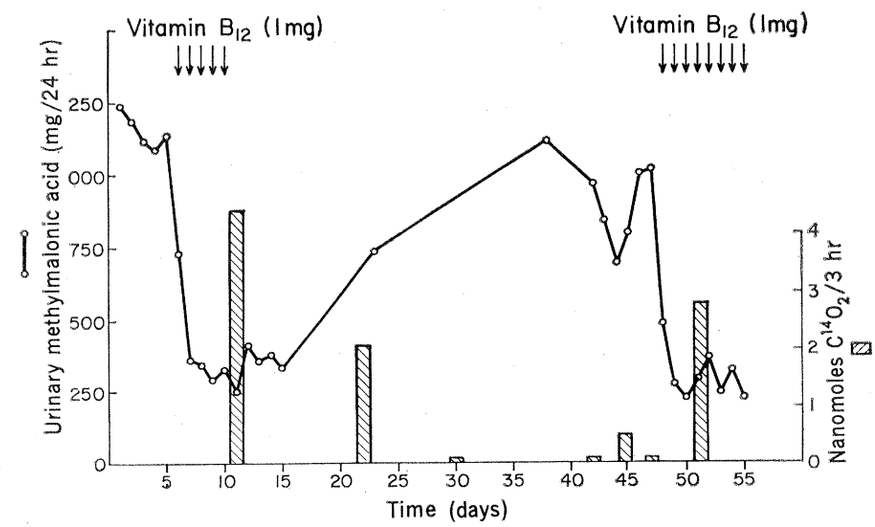

Fig. 3. Effect of parenteral vitamin B_{12} administration on methylmalonic acid excretion (left ordinate) and propionate-3-C^{14} oxidation by isolated leukocytes (right ordinate); nanomoles of $C^{14}O_2$ per 10^8 cells every 3 hours in a patient with methylmalonic aciduria. Each arrow represents single daily injection of vitamin B_{12}. Increasing the amount of vitamin B_{12} administered to 4 mg/day failed to depress urinary methylmalonic acid further.

These effects of vitamin B_{12} are significant for three reasons. First, they provide evidence (albeit indirect) for a defect in methylmalonyl CoA isomerase, because it is the only enzyme in the involved pathway which requires a cobamide coenzyme. Second, they demonstrate a vitamin B_{12} "dependency" state in man, as distinguished from vitamin B_{12} deficiency. There are precedents in microbial and mammalian systems for such coenzyme dependency. Bonner *et al.* (*8*) described tryptophan synthetase mutants in *Neurospora crassa* which could convert indoleglycerolphosphate to indole only in the presence of added pyridoxal phosphate, a cofactor for tryptophan synthetase. They suggested that the mutation affected that portion of the tryptophan synthetase genome concerned with catalytic activities involving pyridoxal phosphate.

Frimpter (see *9*) reported that administration of vitamin B_6 to patients with cystathioninuria, an inborn error due to a defect of a pyridoxal phosphate-dependent enzyme, cystathionase, reduced cystathionine excretion. He also noted that addition of pyridoxal phosphate in vitro enhanced the cystathionase activity of liver homogenates from patients with cystathioninuria. Similar findings in vivo and in vitro have been reported by Tada and associates (*10*) in patients with xanthurenic aciduria, an inborn error due to a mutation of a B_6-dependent enzyme. These and other syndromes which respond to vitamin B_6 administration (*11*) are not due to vitamin B_6 deficiency and may reflect apoenzyme mutations which reduce their affinity for pyridoxal phosphate.

In like manner, the observed vitamin B_{12} dependency in our patient can be explained if the mutant methylmalonyl CoA isomerase apoenzyme has

a very low affinity for its cobamide coenzyme and if, at maximum concentration of coenzyme (as after administration of 1 mg of vitamin B_{12} daily), appreciable binding of coenzyme and of substrate occur with partial restoration of catalytic function. The mechanism of such a proposed apoenzyme-coenzyme interaction is obscure but might involve interaction of enzyme subunits or an allosteric effect on a single polypeptide. The observed vitamin B_{12} dependency could also reflect abnormalities in vitamin B_{12} transport or conversion of the vitamin to its active coenzyme form, but this possibility seems much less likely.

Finally, vitamin B_{12} administration may be useful therapeutically in patients with methylmalonic aciduria, which may not be a rare inborn error, the disorder having been described in three countries in less than 1 year (*3, 4, 12*). This disorder could be detected by urinary screening techniques in newborns, and treatment with large doses of vitamin B_{12} could prevent the characteristic and potentially lethal episodes of keto-acidosis.

LEON E. ROSENBERG
ANNE-CHARLOTTE LILLJEQVIST
YUJEN EDWARD HSIA
Departments of Pediatrics and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

References and Notes

1. E. V. Cox and A. V. White, *Lancet* **1962-II**, 853 (1962); L. A. Barness, D. G. Young, R. Nocho, *Science* **140**, 76 (1963).
2. E. R. Stadtman, P. Overath, H. Eggerer, F. Lynen, *Biochem. Biophys. Res. Commun.* **2**, 1 (1960); R. M. Smith and K. J. Monty, *Biochem. Biophys. Res. Commun.* **1**, 105 (1959); J. R. Stern and D. L. Friedman, *ibid.* **2**, 82 (1960); P. Lengyel, R. Mazumder, S. Ochoa, *Proc. Nat. Acad. Sci. U.S.* **46**, 1312 (1960).
3. V. G. Oberholzer, B. Levin, E. A. Burgess, W. F. Young, *Arch. Dis. Child.* **42**, 492 (1967); O. Stokke, L. Eldjarn, K. R. Norum, J. Steen-Johnsen, S. Halvorsen, *Scand. J. Clin. Lab. Invest.* **20**, 313 (1967).
4. L. E. Rosenberg, A.-C. Lilljeqvist, Y. E. Hsia, *New Engl. J. Med.* **278**, 1319 (1968).
5. R. Robineaux, J. Lebrun, R. Kourilsky, A. Delauny, *Ann. Inst. Pasteur* **77**, 710 (1949); N. Dioguardi, A. Agostino, B. Lomanto, A. Piccaluga, *Haematol. Lat.* **5**, 99 (1962). Equal volumes of heparinized blood and 1.0 percent polyvinyl pyrollidone (average molecular weight 360,000) in 0.9 percent NaCl were mixed in a syringe and allowed to sediment for 45 minutes. The plasma, containing many leukocytes, was expressed into round-bottom test tubes and centrifuged at 140*g* for 15 minutes. The supernatant was decanted, and the cells were resuspended in 0.8 percent NH_4Cl with a Pasteur pipette. After 5 minutes, the tubes were centrifuged at 100*g* for 15 minutes, and the supernatant was decanted. The pellet was suspended in Krebs-bicarbonate buffer (*p*H 7.4), centrifuged at 100*g* for 15 minutes, and finally resuspended in Krebs-bicarbonate buffer. (All procedures at room temperature in nonwettable glass or plastic; all reagents contained 0.45 mg of *D*-glucose per milliliter.)
6. L. E. Rosenberg, A. N. Weinberg, S. Segal, *Biochim. Biophys. Acta* **48**, 500 (1961); R. L. Stjernholm, *J. Bacteriol.* **93**, 1657 (1967).
7. A. J. Giorgio and G. W. E. Plaut, *J. Lab. Clin. Med.* **66**, 667 (1965). Urine samples were diluted 1:40 to 1:200 with 0.1*N* HCl prior to analysis. After addition of the buffer and diazotized *p*-nitroaniline, the reaction mixtures were heated to 94°C for 3 minutes. The tubes were then stoppered and plunged into an ice bath for 1 minute before the addition of 3*N* NaOH. The tubes were held at room temperature for 10 minutes and optical density was determined. The rapid cooling and 10-minute wait were required for a linear standard curve and proportional color development.
8. D. M. Bonner, Y. Suyama, J. A. DeMoss, *Fed. Proc.* **19**, 926 (1960).
9. G. Frimpter, *Science* **14**, 1095 (1965); ———, in *Amino Acid Metabolism and Genetic Variation*, W. L. Nyhan, Ed. (McGraw-Hill, New York, 1967), p. 315.
10. K. Tada, Y. Yokoyama, H. Nakagawa, T. Yoshida, T. Arakawa, *Tohoku J. Exp. Med.* **93**, 115 (1967).
11. C. R. Scriver, *Amer. J. Dis. Child.* **113**, 109 (1967).
12. G. Morrow, L. A. Barness, V. H. Auerbach, A. M. DiGeorge, *Abstr. Soc. Pediat. Res.* **38**, 20 (1968).
13. Supported by grants from the John A. Hartford Foundation and the USPHS (AM 09527), by a research career development award to L.E.R. (AM 28087), and NIH grant FR 00125. We thank Dr. R. Stjernholm, for suggestions and discussions.

5 August 1968

Frame Shift Mutations near the Beginning of the Lysozyme Gene of Bacteriophage T4

Abstract. *A pair of frame shift mutations in the lysozyme gene of bacteriophage T4 results in the substitution of a glutamyl-tyrosyl sequence for the asparagine residue that is the penultimate amino-terminal amino acid in the lysozyme of the wild-type strain. One of the mutations has been identified as the insertion of two bases, the other as the insertion of a single base.*

Earlier (*1–3*) we compared amino acid sequences of lysozyme produced by strains of phage T4 carrying pairs of frame shift mutations to that of the wild-type strain. In all cases we found a sequence of amino acids changed; our results thus confirm Crick's (*4*) theories regarding the general nature of the genetic code. In addition, we have been able to identify the mutations and to assign codons, proposed on the basis of studies in vitro (*5, 6*), to the amino acids within the regions of change.

We now describe the analysis of a strain carrying two frame shift mutations near the amino-terminal end of the lysozyme molecule. The analysis

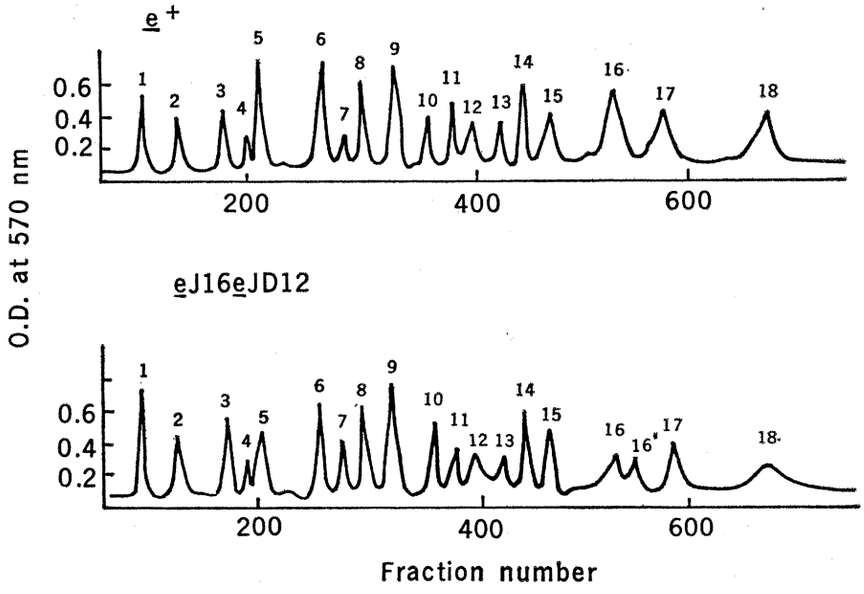

Fig. 1. Elution patterns of tryptic digests of e^+ and *e*J16*e*JD12 lysozymes. Tryptic digests of lysozyme oxidized by performic acid were applied to a Dowex-50 column (0.9 by 150 cm) and eluted by a gradient of pyridine acetate buffer of increasing *p*H and pyridine concentration by means of an eight-chamber Varigrad apparatus (*1*). Fractions of 4.0 ml were collected, and portions from alternate tubes were hydrolyzed with alkali and treated with ninhydrin; the absorbance at 570 nm was measured.