in others the red blood cell count remained below normal. It appeared that in these animals, the transplantation had only been partially successful. However, a possible explanation suggested itself upon electrophoresis of the hemoglobins.

Mice of the W-series have a hemoglobin which on electrophoresis gives a single band. The hemoglobin pattern in CBA mice is diffuse and comprises two well-defined bands. The major, fast component of the hemoglobin corresponds to the single band of the W-series.

Electrophoresis of the hemoglobins of the ten mice revealed that the animals fell into two categories. Six mice had the characteristic two-banded CBA pattern, whereas four had the single W-type pattern (Fig. 1). When the two series of data were compared it was found that the six mice which had the CBA hemoglobin were the animals whose blood picture had become completely normal, and the four animals with the W-type hemoglobin were those in which the red blood count was lower (Table 1). There was no overlap between these two groups, the red blood counts being 11.17 to 12.68 $\times$ 10^6 for the CBA hemoglobin group and 9.89 to 10.55 $\times$ 10^6 for the W hemoglobin group. The difference between the two groups was significant ($P = .01$ to .001, by t-test). The red blood counts of the W-type hemoglobin group corresponded closely to those of W^vw mice—9.16 to 10.80 $\times$ 10^6 ($P = .3$ to .2).

The single animal that survived the CBA spleen alone also showed a permanent change in the peripheral blood picture to one typical of a normal mouse and had hemoglobin of the CBA electrophoretic mobility.

Thus the injected spleen cells have become implanted on the host animals,

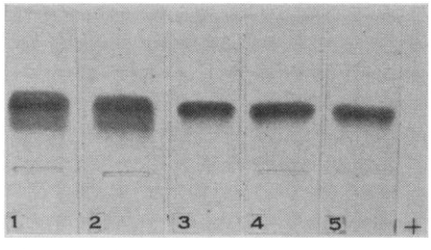

Fig. 1. Electrophoresis of hemoglobins of CBA strain, W^vW^v, and W^vw mice, and W^wW^w mice treated with CBA/W^vw spleen cell mixture. 1, CBA; 2, W^wW^w, treated with CBA/W^vw spleen; 3, W^wW^v; 4, W^wW^v, treated with CBA/W^vw spleen; 5, W^vw.

and the erythropoietic elements are functioning normally to produce erythrocytes. However, it appears that when a mixture of homologous CBA cells and isologous W^vw cells was offered, it was a random chance whether the CBA cells or the W^vw cells implanted. In the case of the animals which showed the CBA diffuse type of hemoglobin, it cannot be stated with certainty that there is no W^ww tissue functioning, but in the remaining four mice there is no doubt concerning the absence of CBA erythropoietic cells.

When Bernstein and Russell (*5*) first transplanted hematopoietic tissue into the W-series anemics, they suggested that the tissue implanted and continued to function according to its genotype. The present work may well provide evidence to support this hypothesis. The heterozygotes W^ww of the W-series mice have an anemia, although it is less severe than that possessed by the W^vW^w mice. They also have a very slight macrocytosis. The W^vW^w injected mice, which on electrophoretic evidence had W^vw tissue implanted, had a slightly anemic blood picture and slight macrocytosis of the red cells, when compared with the hematologically completely normal picture of the CBA-treated individuals. In these two cases, therefore, there seems to be readily observable proof of the transplanted cells functioning according to their genotype.

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31 October 1966

Biosynthesis of DNA by Isolated Mitochondria: Incorporation of Thymidine Triphosphate-2-C^{14}

Abstract. *Thymidine triphosphate-2-C^{14} and other deoxynucleoside triphosphates are incorporated into the DNA of isolated mitochondria from rat liver cells. The reaction is partially dependent on the other deoxynucleoside triphosphates and proceeds in the virtual absence of bacteria. The product has the properties of mitochondrial rather than nuclear DNA, and end-group studies indicate labeling of the interior of the molecule.*

The control of mitochondrial structure and function by cytoplasmic (*1*) as well as by nuclear (*2*) genes, the "nonconservative" replication of certain structural elements of mitochondria (*3*), and the presence in mitochondria of DNA (*4*), RNA (*5*), and a protein biosynthesis system (*6*) point to the semi-independence of these organelles. We now report on the incorporation of TTP-2-C^{14} (*7*) into the DNA of isolated mitochondria from rat liver, a reaction which may represent DNA replication.

The mitochondria were isolated according to the method of Schneider and Hogeboom (*8*) except that 0.001*M* EDTA, *p*H 7.0, was included in the isolation medium and all mitochondrial pellets were washed four times. In early experiments, mitochondria were further purified by centrifugation through 1*M* sucrose at 8500*g* for 30 minutes. In later experiments, sedimentation-velocity centrifugation in a continuous sucrose density gradient was used. In a gradient of 0.6 to 1.5*M* sucrose, sedimentation at 8500*g* for 20 minutes resulted in a somewhat diffuse band at a sucrose concentration averaging about 1.2*M*; in a gradient of 0.3 to 0.6*M* sucrose at 2543*g* for 20 minutes, banding occurred at about 0.5*M* sucrose. Immediately after the sedimentation, the mitochondrial band was carefully removed with a Pasteur pipette, and this material was diluted with water to a sucrose concentration of 0.25*M*. In the case of the more strongly hypertonic gradient, this dilution was performed as quickly as possible to minimize the period of contact of the mitochondria with the hypertonic sucrose. (Experiments done for another purpose showed that

incubation of the mitochondria in 1.5*M* sucrose for 90 minutes completely abolished incorporation.) After dilution, the mitochondria were again sedimented at 8500*g* for 10 minutes and suspended in 0.25*M* sucrose.

Labeled DNA was isolated (*9*) in the presence of carrier calf thymus DNA, treated with pancreatic ribonuclease, and dialyzed against 0.15*M* NaCl–0.015*M* sodium citrate, *p*H 7.5. For buoyant-density studies, the DNA was isolated without carrier and purified by preparative CsCl density-gradient centrifugation (*10*). The DNA band was recovered for buoyant-density and radioactivity determinations by collection of six-drop fractions from the gradient. The DNA was denatured and renatured as described by Corneo *et al.* (*11*).

To determine the extent of labeling in the interior of the molecule, DNA was incubated with micrococcal nuclease and spleen phosphodiesterase (*12*). The resulting 3′-monophosphates and free nucleosides released from the 3′-end of the molecule were separated by paper chromatography (*13*).

Incubation of rat liver mitochondria for a 2-hour period resulted in the linear incorporation of TTP-2-C^{14}, in an amount corresponding to the synthesis of 0.5 to 1 percent of the total mitochondrial DNA. This figure is based on a DNA content of 0.25 μg of DNA per milligram of mitochondrial protein, a value which we obtain from mitochondrial preparations further purified successively by density-gradient sedimentation velocity and isopycnic centrifugation. Although all four nucleoside triphosphates are incorporated (*14*), the requirements for incorporation of only two have been studied. The incorporation of TTP-2-C^{14} or dATP-8-C^{14} was partially dependent on the presence of the other three nucleoside triphosphates (Table 1). Also the incorporation was inhibited 80 to 90 percent by actinomycin D at concentrations that inhibit DNA polymerase action (*15*), but it was only inhibited 10 to 15 percent by deoxyribonuclease.

The rate of synthesis of both protein and DNA by isolated mitochondria, as measured by incorporation of precursors, is sluggish; and therefore results of incorporation experiments must be scrutinized critically. In our experiments it was imperative to show that the labeled product is indeed DNA, to locate the position of the label in the DNA molecule insofar as possible, and to establish that the labeled DNA species is indeed mitochondrial.

Evidence that the labeled product is DNA comes from experiments in which the isolated radioactive material became completely soluble after incubation with pancreatic deoxyribonuclease, but was resistant to both alkaline hydrolysis and incubation with pancreatic ribonuclease. Further evidence comes from the coincidence of the DNA (buoyant density) and radioactivity peaks on CsCl isopycnic centrifugation.

The position of the label has not been completely elucidated. However, in an experiment in which 82 percent of the radioactivity was recovered, chromatographic separation of the 3′-mononucleotides and free nucleosides released from the 3′-end of the molecule by complete digestion of the labeled DNA with micrococcal nuclease and spleen phosphodiesterase revealed that there was no radioactivity in any of the mononucleotides except 3′-TMP. Comparison between the amount of label of 3′-TMP and free thymidine released from the 3′-end of the molecule showed that virtually all the label was in the 3′-TMP. From these results it is unlikely that a spurious terminal labeling reaction is responsible for the incorporation.

The decline in the rate of incorporation after 2 hours suggests that the labeling did not result from contaminating bacteria; however, additional studies were done to rule out this possibility. Separation of the bacteria from the mitochondria, after incubation, by isopycnic sucrose-density gradient centrifugation, showed no radioactivity whatsoever in the bacterial fraction. Furthermore, experiments with germ-free animals and aseptic techniques revealed that although the number of contaminating bacteria were reduced from 2×10^5 to 250 organisms per milliliter of incubation medium (no anaerobes were detected), no inhibitory effect whatsoever was found on the rate or extent of incorporation.

Several lines of evidence argue against the incorporation being effected by a contaminating nuclear system. Phase-contrast microscopy showed that there were no intact nuclei. Electron microscopy failed to reveal definitive evidence of nuclear fragments, al-

Table 1. Incorporation (μmole/hr per 10 mg of mitochondrial protein) of deoxynucleotides into the DNA of isolated mitochondria from rat liver cells. The complete system contained the following concentrations of reagents: 4 m*M* KCl, 7 m*M* $MgCl_2$, 5 m*M* KH_2PO_4, 2 m*M* ATP, 40 m*M* nicotinamide, 7.5 m*M* pyruvate, 7.5 m*M* succinate, 0.45 m*M* malate, 0.015 m*M* of each unlabeled nucleotide, 0.004 m*M* labeled nucleotide and 10 mg of mitochondrial protein per milliliter at a final *p*H of 7.4. The specific activities were 30.3 mc/mmole for TTP-2-C^{14} and 17.15 mc/mmole for dATP-8-C^{14}. The incubations were carried out at 37°C in final volumes of 1 to 5 ml. All samples counted were from 5 to 10 times background. The data given represent minimum values since no account has been taken of possible dilution of the labeled nucleoside triphosphates by endogenous pools.

Reaction mixture	Incorporation
Complete (TTP-2-C^{14})	3.05
— dATP	1.80
— dCTP	1.80
— dGTP	1.65
+ Actinomycin D (200 μg/ml)	0.66
Complete (TTP-2-C^{14})	4.70
+ Actinomycin D (200 μg/ml)	0.45
+ Deoxyribonuclease (50 μg/ml)	4.18
Complete (TTP-2-C^{14})	4.81
— dATP, dCTP, dGTP	3.11
+ Deoxyribonuclease (50 μg/ml)	4.05
Complete (dATP-8-C^{14})	9.14
— dCTP, dGTP, TTP	6.28

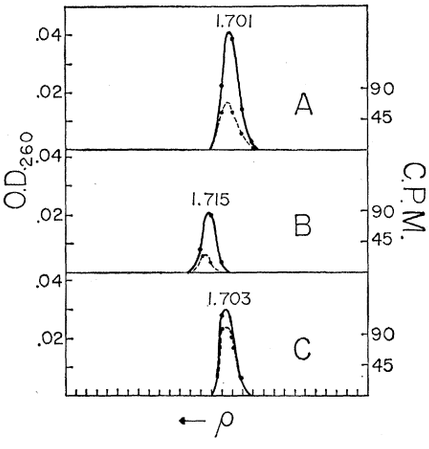

Fig. 1. Preparative isopycnic centrifugation of mitochondrial DNA. Samples A, B, and C, which represent native, denatured, and renatured DNA, respectively, were taken in equal volumes from the same preparation of mitochondrial DNA and were centrifuged simultaneously. Twenty-eight fractions were obtained from each tube, and they are represented in the divisions on the abscissa. Buoyant densities were determined directly from the refractive indices of the peak fractions and are indicated over the peaks. These values (as well as our values for nuclear DNA) agree with those obtained by others from analytical ultracentrifuge data (*11*). Control experiments indicate that the variation in recovery of OD units and of radioactivity in the three different runs is related, at least in part, to the strong and preferential adsorption of denatured DNA to the walls of cellulose nitrate centrifuge tubes (*19*); much of the lost radioactivity could be accounted for when sections of centrifuge tubes were counted in a Geiger counter.

though occasional unidentifiable structures were present (*12*). Pancreatic deoxyribonuclease, which can digest nuclear DNA almost entirely even in the presence of mitochondria (*16*), inhibited the incorporation only 10 to 15 percent. Preliminary incubation of the mitochondria alone in strongly hypertonic sucrose for more than short periods of time (1.5*M* for 90 minutes), a procedure which alters mitochondrial structure but would not be expected to affect nuclear fragments or free DNA polymerase, prevented the incorporation entirely. (The incorporation process is stable to such prior incubation when isotonic sucrose is used.) Finally, when the changes in buoyant density of DNA upon denaturation and renaturation are followed by CsCl isopycnic centrifugation (Fig. 1), the isolated mitochondrial DNA, in contrast to the results of control experiments with nuclear DNA (not shown), is renaturable after heat denaturation, and the radioactivity peaks remain coincident with the DNA (OD 260 mμ) peaks both after denaturation and upon renaturation (Fig. 1). Ability to renature has been considered a criterion for distinguishing between mitochondrial and nuclear DNA (*11, 17*).

In the light of these findings, we believe that mitochondria from rat liver cells not only contain a unique DNA (*18*), but that the mitochondria themselves also contain a mechanism for incorporating nucleotides into this DNA. Whether this incorporation reflects the presence of a mechanism for DNA replication, a repair process, or some as yet unknown phenomenon is a problem for future study.

Note added in proof: After this report was submitted, we learned that others, using mitochondrial systems, have obtained incorporation of DNA precursors into DNA (*20, 21*); these workers, however, have not yet established that the labeled DNA is, in fact, mitochondrial DNA.

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Phosphate Uptake in an Obligately Marine Fungus: A Specific Requirement for Sodium

Abstract. *Phosphate uptake in the obligately marine fungus* Thraustochytrium roseum *is maximally stimulated by sodium chloride in a range of concentrations (0.2 to 0.4 molar) similar to those commonly encountered in littoral habitats. The effectiveness of sodium chloride for phosphate transport extends beyond its osmotic function and can be attributed specifically to sodium. Increases in respiration in the presence of the salt can be ascribed primarily to an osmotic effect.*

Sea water or NaCl is required for the culture of many marine bacteria and fungi (*1*, *2*). Recent studies suggest that the growth requirement for NaCl is specific for these organisms and may be independent of its osmotic function (*3*). Payne (*4*) related the importance of sodium to its function in facilitating substrate penetration, and MacLeod *et al.* (*5*) provided evidence of its role in the oxidation of exogenous substrate. They showed that those substrates supporting optimal growth were also those whose oxidation was stimulated by sodium. Experiments with cell-free extracts have been unsuccessful since NaCl was not related to the activation of specific enzymes (*5*). Attention has therefore focused upon the possible role of NaCl in transport phenomena. Using substrate analogs that could not be metabolized, Drapeau and MacLeod (*6*) concluded that, in marine bacteria, the primary function of sodium was to permit the entry of these compounds into the cell. In view of these findings, *Thraustochytrium roseum*, an obligately marine, nonfilamentous phycomycete (*2*), seemed uniquely suited for examination of the effect of NaCl on other processes such as ion transport. The utility of this fungus lies in its high degree of endogenous respiration, which is unaffected by exogenous substrates (*7*). Consequently, the influence of NaCl on ion transport could be studied in the absence of external energy sources, which might influence ion uptake.

Thalli of *T. roseum* were grown for 4 days at 20°C in a chemically defined medium (*8*). The cultures were maintained on a reciprocal shaker under diffuse incandescent light (8×10^4 erg/cm^2). Cells from cultures in the log phase of growth were centrifuged and resuspended in the same volume

Table 1. Influence of cations and sucrose on phosphate and oxygen uptake by *Thraustochytrium roseum*. The reaction mixture contained: tris-HCl (0.1*M*, *p*H 7.8), KH_2PO_4 (0.001*M*, *p*H 7.8), cells (210 μg of protein per milliliter), and salts as indicated. Oxygen uptake was linear during the 4-hour experimental period.

Additions	Concentration (mole/liter)	Phosphate uptake (μmole/2 hr)	Phosphate uptake (μmole/4 hr)	Oxygen uptake (μmole/1 hr)
None		0.00	0.07	6.52
LiCl		0.09	0.11	5.45
NaCl	0.2	0.32	1.01	9.38
KCl	0.2	0.04	0.10	8.04
RbCl	0.2	0.00	0.00	6.92
$MgCl_2$	0.067	0.23	0.40	8.22
Sucrose	0.2	0.00	0.27	8.08
Sucrose	0.4	0.09	0.26	6.83