shaved skin of the back with 200 μg of lead acetate, cerium chloride, or potassium permanganate, or 2 mg of calcium chloride, in 0.5 ml of distilled water. Preliminary experiments had shown that at these dosages all the agents tested produce calcified disks having a mean diameter of about 10 mm.

Each rat received one of these calcergens at one point alone and, at a distance of 2 to 3 cm, conjointly with a test substance. In the cases of lead acetate, cerium chloride, and calcium chloride, the calcergen and the test substance were mixed in 0.5 ml of water, whereas potassium permanganate (which caused precipitates with many of the test substances) had to be injected 3 minutes before the potential inhibitor (each in 0.25 ml of water) at the same spot.

The animals were kept on Purina Fox Chow during the experiment and killed with chloroform on the 5th day. The diameters of the resultant disks were averaged, and the calcific nature of the deposits was subsequently verified histochemically (von Kóssa and chloranilic acid tests).

The histologic details and the statistical evaluation of the data will be published elsewhere, but, since in most instances the calcification was either maximal or nil, the results can be easily summarized as follows:

At doses of 500 μg there was no inhibition by AgCl, $CeCl_3$, CsCl, $CuCl_2$, $HgCl_2$, KCl, KI, LiCl, $MgCl_2$, $MnCl_2$, NaCl, Na_2PtCl_6, NH_4Cl, $NiCl_2$, RbCl, Re_2O_7, SeO_2, $SrCl_2$, or $TlCl_3$, each of which had been shown not to act as a calciphylactic challenger in appropriately sensitized rats (*1*). On the other hand, the following verified calciphylactic challengers completely, or virtually completely, inhibited the calcification normally induced by any of our four calcergens: $AlCl_3$, $CrCl_3$, $FeCl_2$, $FeCl_3$, $SnCl_2$ (each at a dose of 500 μg), aluminium dextran (equivalent to 9.7 mg of aluminium), chromium dextran (9.85 mg of chromium), ferric dextran (6.25 mg of iron), ferric dextrin (2.5 mg of iron), ferric oxysaccharate (2.5 mg of iron), and ferric sorbitol (2.5 mg of iron).

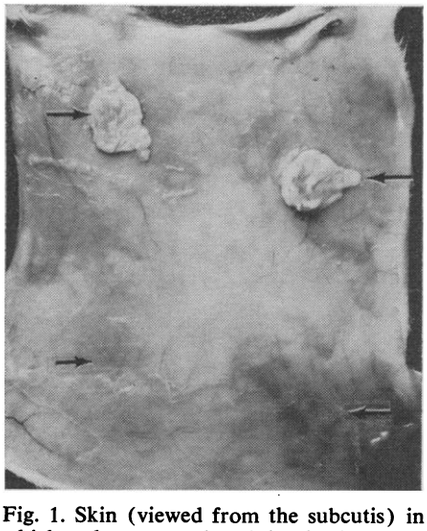

Fig. 1. Skin (viewed from the subcutis) in which calcergy produced by lead acetate (top left) and potassium permanganate (top right) is inhibited by ferric dextran given conjointly with the same agents (bottom left and bottom right, respectively).

Figure 1 shows macroscopically the skin of a rat in which the calcifications that are normally produced by lead acetate and $KMnO_4$ were inhibited by ferric dextran.

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Interconversions of Mitochondrial Pyridine Nucleotides

Abstract. *Among the factors that can govern the relative rates of mitochondrial oxidation of isocitrate by means of the isocitrate dehydrogenases linked to diphospho- and triphosphopyridine nucleotides are the intramitochondrial concentrations of these nucleotides. Yeast mitochondria contain enzymes that can alter the ratio of these pyridine nucleotides by interconverting them. A diphosphopyridine nucleotide kinase catalyzes the formation of triphosphopyridine nucleotide from diphosphopyridine nucleotide and adenosine triphosphate; a phosphatase converts triphosphopyridine nucleotide to diphosphopyridine nucleotide. Both reactions are more active with the reduced forms of the pyridine nucleotide coenzymes. These studies suggest that the activity of the mitochondrial triphosphopyridine-nucleotide–linked isocitrate dehydrogenase may be regulated by a cyclic system of pyridine-nucleotide interconversions involving triphosphopyridine nucleotide synthesis and breakdown.*

The oxidation of isocitrate to α-ketoglutarate and CO_2 in mitochondria can proceed by either TPN-linked or DPN-linked isocitrate dehydrogenase (TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide). These two pathways have been demonstrated with mitochondria from tissues of rat, pigeon, and locust (*1*, *2*), and from guinea pig heart (*3*) and yeast (*4*). Although the regulation of the DPN-linked pathway of isocitrate oxidation has been described (*5*, *6*), little is known of the mechanism which controls the TPN-linked pathway. We have suggested that the intramitochondrial concentration of TPN might be responsible for regulating the TPN-linked pathway (*4*). This was based on kinetic data which indicated that significant amounts of TPN are either not present in isolated yeast mitochondria or not accessible to the TPN-linked isocitrate dehydrogenase.

Intact yeast mitochondria were prepared as previously described (*4*). Pyridine nucleotides were extracted with perchloric acid (*7*) after oxidation with 5-ethylphenazinium ethyl sulfate and air (*8*). This procedure yields the total content of the pyridine nucleotides without regard to their original state of reduction. The coenzymes were then assayed enzymatically: DPN with ethanol and alcohol dehydrogenase from yeast, and TPN with isocitrate and TPN-specific isocitrate dehydrogenase from pig heart (*9*). Protein was measured according to the method of Lowry *et al.* (*10*).

All incubations were conducted in test tubes (18 by 65 mm) immersed in a 30°C water bath, and the reactions were initiated by introduction of mitochondria. Mixing was done with small magnetic stirring bars propelled by a submerged motor; and the vessels were exposed to air, except when reduced pyridine nucleotides were present. In the latter cases, the reaction mixtures were kept anaerobic with a stream of water-saturated nitrogen to prevent oxidation of the reduced pyridine nucleotides by the mitochondrial preparations.

Direct analysis of mitochondrial suspensions gave the following values for the pyridine nucleotides: DPN + DPNH = 2.71 ± 0.61; TPN + TPNH = 0.43 ± 0.18 nmole per milligram of

mitochondrial protein. Each figure is the mean ± standard deviation of 24 determinations, representing six mitochondrial preparations. The amount of total DPN is comparable to that found in rat liver mitochondria; however, the total TPN is less than 12 percent of the amount found in rat liver mitochondria (*11*). This lends support to our finding (*4*) that, in isolated yeast mitochondria, TPN appears to be present in rate-limiting quantities.

The ability of yeast mitochondria to convert DPN into TPN is shown in Fig. 1. When DPN is the substrate, TPN formation occurs only to a limited extent before the reaction stops. With DPNH, however, the reaction proceeds more rapidly and to a much greater extent. Although the actual products in each case have not been determined, they are probably TPN and TPNH when the substrates are DPN and DPNH, respectively.

Diphosphopyridine nucleotide kinase, the enzyme that catalyzes the synthesis of TPN, was originally isolated from autolyzates of yeast by Kornberg (*12*), who showed that the enzyme was able to phosphorylate both DPN and DPNH. Although the intracellular distribution of this enzyme is not known, our work indicates that the level of DPN kinase in yeast mitochondria is sufficient to phosphorylate the endogenous DPNH in a relatively short time. Vignais and Vignais (*13*) demonstrated that rat liver mitochondria can also convert DPN into TPN.

The conversion of DPNH to TPNH in mitochondria by a mechanism that requires ATP superficially resembles the energy-linked transhydrogenase system (*14*) in which ATP or a high-energy intermediate generated during oxidative phosphorylation catalyzes the transfer of hydrogen from DPNH to TPN. Energy-linked transhydrogenase does not result in a net conversion of one coenzyme to the other (*15*) but mediates, instead, a redistribution of the oxidized and reduced forms of each coenzyme by a process associated with the hydrolysis of a high-energy bond.

The ability of yeast mitochondria to synthesize triphosphopyridine nucleotide suggested that these mitochondria might also regenerate diphosphopyridine nucleotide by hydrolytic cleavage of the 2′-phosphate of TPN and TPNH. Although it is known that alkaline phosphatases are able to convert TPN into DPN (see *16*), to the best of our knowledge, no specific phosphatase for this reaction has been reported as yet.

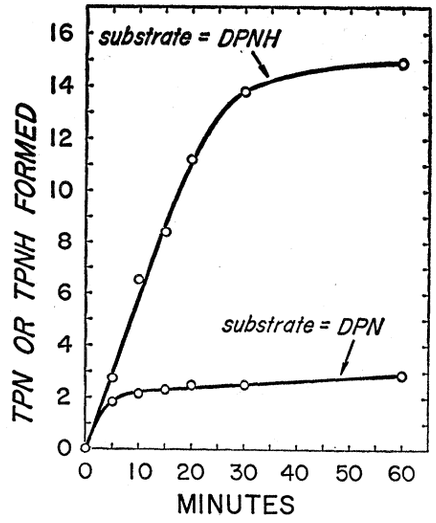

Fig. 1. Formation of triphosphopyridine nucleotide (nanomoles per milligram of protein) from DPN and DPNH. The reaction mixtures contain: 120 m*M* tris chloride, *p*H 7.8; 2.4 mg of crystalline bovine serum albumin per milliliter; 10 m*M* $MgCl_2$; 10 m*M* KCl; 3 m*M* ATP; 10 m*M* trisodium phosphoenolpyruvate; 10 units of pyruvate kinase (Sigma) per milliliter; 3 m*M* DPN or DPNH; 4 m*M* sodium deoxycholate, and 2.7 mg of mitochondrial protein per milliliter. Values are corrected for endogenous TPN.

We found that yeast mitochondria catalyzed the hydrolysis of TPN and TPNH to diphosphopyridine nucleotide at rates comparable to the DPN-kinase reaction. These reactions proceed more rapidly with TPNH than with TPN, and they are linear for at least 60 minutes.

The pyridine-nucleotide interconversions described here may be a means for controlling the activity of the TPN-linked isocitrate dehydrogenase in yeast mitochondria. The DPN-linked pathway of isocitrate oxidation is essentially an irreversible reaction (*17*), coupled to the respiratory chain. Operation of this pathway results in the formation of DPNH which can be aerobically used for the production of ATP. This pathway in yeast is primarily controlled by feedback from oxidative phosphorylation by the adenine nucleotides: adenosine monophosphate by virtue of its allosteric activation of the dehydrogenase (*5*, *6*), and adenosine diphosphate through its effect on the respiratory chain (*18*). Further control may also be exercised by inhibition of the dehydrogenase by DPNH (*6*). Thus, under energy-saturating conditions, when the levels of ATP and DPNH are increased and those of ADP and AMP are low, the enzyme should be inhibited; conversely, when the levels of ATP and DPNH are low and those of ADP and AMP are increased, this pathway should be activated.

On the other hand, the TPN-linked pathway of isocitrate oxidation is readily reversible (*17*), and because it is not coupled to the respiratory chain (*2*), it is free to operate when the levels of ATP and DPNH are high. Under these conditions, the synthesis of TPNH by the DPN-kinase reaction is favored. The TPN-linked isocitrate dehydrogenase will function to maintain triphosphopyridine nucleotide in the reduced state and to supply essential organic intermediates at a time when the Krebs cycle is otherwise inhibited.

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