addition of vitamin B_{12} *in vitro* were unsuccessful, as were attempts to inhibit with cyanide; however, when 5,6-dimethybenzimidazole-B_{12} coenzyme[5] was used the isomerase activity was completely restored to normal (Table I).

Two experiments were carried out, each in duplicate, involving two normal and two deficient rats. The reaction mixture complete system contained Tris buffer, pH 7.4, 400 μmoles; $MgCl_2$, 10 μmoles; GSH, 10 μmoles; ATP, 10 μmoles; propionyl-CoA[6], 1 μmole; $NaH^{14}CO_3$, 10 μmoles (10^6 counts/min); and buffer extract of acetone-dried rat-liver mitochondria[7,8], containing 18–25 mg protein. The final volume was 1.7 ml. The mixtures were incubated at 28° in an atmosphere of $O_2 + CO_2$ for 1 h and the reaction was stopped by the addition of 0.3 ml 2 *N* $HClO_4$. Direct scintillation counting of acid-deproteinized aliquots gave the total radioactivity incorporated while counting after heating aliquots for 10 min at 100° with a few drops of 3% $KMnO_4$, and removal of excess permanganate by reduction with methanol gave the radioactivity due to succinate, since succinate is stable to this permanganate oxidation, while methylmalonate, as well as malate and fumarate, is oxidized to volatile products. The precipitated MnO_2 was removed by centrifugation before counting of the filtrates. All results are expressed in counts/min/mg protein (enzyme preparation) added.

The data given in Table I clearly indicate that vitamin B_{12} coenzyme is a cofactor for the isomerization of methylmalonyl-CoA to succinyl-CoA.

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Nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*

Biological nitrogen fixation is of fundamental importance to the existence of all living things, yet there has been little progress in understanding the chemistry of the process. Investigations at the enzyme level to define the mechanism have hitherto been blocked by failure to find adequate ways for extracting the enzymes in active

condition. We wish now to report the discovery of reproducible procedures for extracting the nitrogen-fixing enzyme system from *Clostridium pasteurianum*.

Tracer experiments with $^{15}N_2$ and resting cells of *C. pasteurianum* revealed that fixation by intact cells was detectable only in the presence of a metabolic energy source, preferably sodium pyruvate. This information was applied in an investigation of cell-breaking techniques, and two methods were found giving active extracts. In the first method, concentrated suspensions of cells[1] in 0.05 *M* phosphate buffer, pH 6.8, were disrupted in a Hughes press[2] at — 15 to — 35° and transferred while still frozen to assay vessels or centrifuge tubes. In the second and more convenient method, dried cells were autolyzed by shaking them anaerobically in the buffer at 30° and centrifuging to remove debris. The dried cells were prepared by rotating concentrated cell pastes in a "Rinco" evaporator in a water bath at 50° under vacuum. Rigorous control of conditions in these procedures is essential, particularly since the extracts are inactivated by air.

Assays were made by adding 100 mg sodium pyruvate to 3–4 ml of the extracts in a total volume of 8 ml and exposing to $^{15}N_2$ on a shaker at 30°[3]. In 1 h under 60 atom % excess $^{15}N_2$, enrichments ranging from 0.1 to 0.6 atom % excess ^{15}N have been obtained. Activity has persisted for as long as 2 h. That these preparations are free of intact cells has been demonstrated by microscopic examination and ultracentrifugation.

Sodium pyruvate has been the most active substance found for causing fixation in these extracts. Activity could also be detected, though in lesser amounts, with α-ketobutyric acid but not with many other hydrogen donors tested including H_2. Extracts of poor activity could sometimes be improved with supplementary coenzyme A.

The enzyme system appears to be water-soluble since activity was not sedimented by centrifugation at 144,000 × *g* for 2 h. The centrifuged extracts were clear brown solutions that fixed up to 12 μg N/mg protein nitrogen initially present.

The fixation reaction seems to comprise NH_3 synthesis[4] since the $^{15}N_2$ taken up by the extracts could be recovered quantitatively as $^{15}NH_3$. The extracts show a spectral response to nitrogen. The difference spectrum under N_2 against H_2 or A shows absorption in the 300–365 mμ region distinct from oxygen-induced differences which are found at 385–420 mμ.

The techniques applicable to *C. pasteurianum* have not yet been successful for *Azotobacter vinelandii* or *Nostoc muscorum*.

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