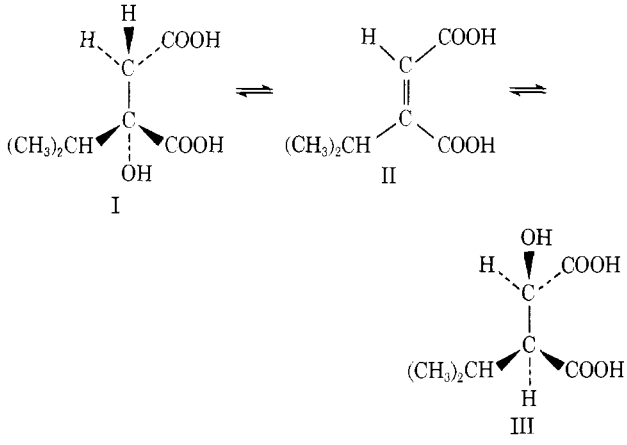
Absolute Configuration of α-Isopropylmalate and the Mechanism of Its Conversion to β-Isopropylmalate in the Biosynthesis of Leucine†

Frank E. Cole, M. G. Kalyanpur, and C. M. Stevens*

ABSTRACT: The absolute configuration of α-isopropylmalate, an intermediate in the biosynthesis of leucine, was established by X-ray crystallographic examination of the monopotassium salt. The configuration of the natural form is (2*S*)-2-hydroxy-2-isopropylsuccinic acid (D_5-α-isopropylmalate). Together with the earlier identification of *erythro*-D_5-β-isopropylmalate, and of dimethylcitraconate as the dehydration product, it can be concluded that the stereochemistry of the α-isopropylmalate ↔ β-isopropylmalate rearrangement is completely analogous to that of the citrate ↔ isocitrate and homocitrate ↔ homoisocitrate systems. Doubly labeled β-isopropylmalate, isolated from cultures of a leucine auxotroph of *Neurospora crassa* incubated with [1-^{14}C]valine and [^{3}H]-acetate, was subjected to chemical degradation to establish the location of ^{3}H. The results indicate that the ^{3}H is predominantly, if not exclusively, on the α carbon. Together with prior evidence, this finding is consistent with stereospecific trans elimination and addition of the elements of water in the enzyme-catalyzed interconversion of α-isopropylmalate and β-isopropylmalate.

Studies of the pathway of biosynthesis of leucine in several microorganisms (*cf.* Gross *et al.*, 1963, and references therein) have demonstrated that the conversion of α-isopropylmalate (I)[1] to β-isopropylmalate (III) is an essential step in the con-



version of α-ketoisovalerate to α-ketoisocaproate, the immediate precursor of leucine.

The mechanism of the isomerization of I to III is of added interest because it is representative of what appears now to be a rather general process for accomplishing the biosynthesis of α-keto acids from the next lower homologs. The classical case is the aconitase-catalyzed conversion of citrate to isocitrate in the principal biosynthetic route to α-ketoglutarate (and glutamate). Other examples include the biosynthesis of α-ketoadipate (and α-aminoadipic acid and lysine) in fungi (*cf.* Chilina *et al.*, 1969, and references therein) and the biosynthesis of α-keto acid (and α-amino acid) precursors of several of the glucosinolates in certain genera of higher plants (*cf.* Underhill, 1968).

Gross *et al.* (1963) have provided strong evidence that in both *Neurospora crassa* and *Salmonella typhimurium* a single enzyme is involved in the conversion of I to III,[2] though in each case the structure of the enzyme is determined by two cistrons. They have purified the isomerase from *Neurospora* and have shown that it catalyzes the equilibration of dimethyl citraconate (II) with I and III. They were unable to demonstrate *in vitro* the conversion of I to III without the formation of II. In addition, II was shown to be utilized for the synthesis of leucine *in vivo* by auxotrophs of *S. typhimurium* which were blocked in the synthesis of I. On the other hand, mutants of *N. crassa* which accumulate I and III in the medium do not appear to accumulate II, despite the fact that the mycelia appear to be impermeable to II. Furthermore, in the only analogous case which has been studied exhaustively, the aconitase-catalyzed rearrangement of citrate to isocitrate in mammalian tissue (*cf.* Rose and O'Connell, 1967, and references therein), there is abundant evidence that free *cis*-aconitate is *not* an obligate intermediate.

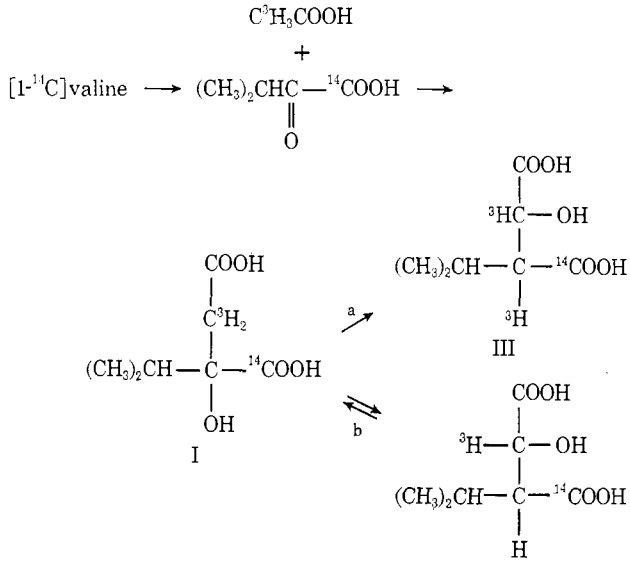
In earlier work on the biosynthesis of leucine (Calvo *et al.*, 1962), in which a leucine auxotroph of *N. crassa* (ATCC No. 14079) was incubated with [1-^{14}C]valine and [^{3}H]acetate and compounds I and III were subsequently isolated from the

† From the Center for Crystallographic Research, Roswell Park Memorial Institute, Buffalo, New York 14203 (F. E. C.), and from the Program in Biochemistry, Department of Chemistry, Washington State University, Pullman, Washington 99163 (M. G. K. and C. M. S.). *Received March 1, 1973.* Supported by Research Grants GM 15787 and AI 01111 from the National Institutes of Health, U. S. Public Health Service.

[1] In the earlier literature, these compounds were referred to as 2-isopropylmalate and 3-isopropylmalate or as β-carboxy-β-hydroxyisocaproic acid and α-hydroxy-β-carboxyisocaproic acid. The nomenclature used here is that agreed upon by other workers in this area. The absolute configuration of the natural β-isopropylmalate has been shown (Calvo *et al.*, 1964) to be (2*R*,3*S*)-2-hydroxy-3-isopropylsuccinic acid (*erythro*-D_5-β-isopropylmalate)).

[2] Recent articles on the isolation and characterization of isopropylmalate isomerase from bakers yeast and from *Neurospora crassa*, as well as methods for the isolation and determination of α- and β-isopropylmalate, are contained in *Methods Enzymol. 17A*, 782, 786, 791 (1970).

medium, the two products were found to have essentially the same ratio of tritium to carbon-14. This result would be consistent with the intramolecular shift of a proton from the α carbon to the β carbon without solvent exchange (path a), as



has been established in the aconitase system. Alternatively, the equal labeling of the two compounds could result from stereospecific interconversion of I and III, by any mechanism which permitted eventual complete exchange of *one* hydrogen on the methylene carbon of I (path b). The first hypothesis would predict the retention of tritium in the β position of III while in the latter case the β hydrogen of III would contain no tritium.

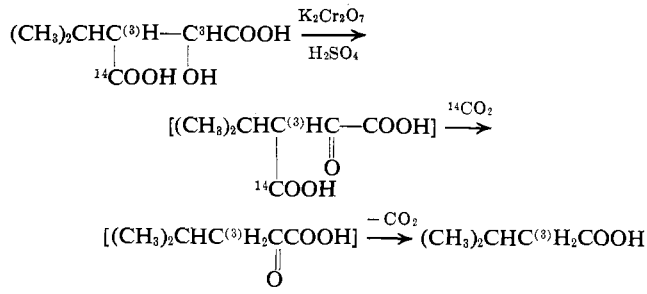
The present work has been directed to the elucidation of the stereochemistry of compound I and of its rearrangement to III.

Materials and Methods

Optically active α-isopropylmalate was isolated from leucine auxotrophs of *N. crassa* as described by Calvo *et al.* (1962). For X-ray diffraction studies, the crystalline monopotassium salt was prepared by dissolving 1 mmol (0.18 g) of the free acid in 10 ml of 0.1 N potassium hydroxide solution. This solution produced rectangular shaped crystals upon evaporation.

A crystal was selected and shaped to the dimensions 0.09 × 0.13 × 0.27 mm and used in this investigation. The space group determination, unit cell dimensions, and intensity measurements were performed on a G. E. XRD-6 manual diffractometer equipped with goniostat. The stationary crystal–stationary counter technique (Furnas and Harker, 1955) was employed in the measurement of the intensities. Monochromatization of the Cu Kα radiation was effected by an almost perfectly balanced pair of Ross filters: Ni and Cu. The absolute configuration was established by the *R*-factor ratio method of Hamilton (1965).

The doubly labeled β-isopropylmalate was isolated in a previous experiment (Calvo *et al.*, 1962). A portion of the labeled material was diluted with 100 mg of the nonlabeled synthetic racemate A and chromatographed on silicic acid using chloroform–*n*-butyl alcohol. Fractions comprising the peak for racemate A were combined and evaporated and the product was recrystallized. The purified material was then subjected to oxidation by $K_2Cr_2O_7$–H_2SO_4 to convert it to isovaleric acid.



A second aliquot of doubly labeled β-isopropylmalate was diluted with 100 mg of synthetic racemate A and recrystallized three times from ether–ligroin to a constant specific activity of ~2000 cpm/mg. This material was then acetylated with acetyl chloride (Calvo *et al.*, 1964) and the mixture pyrolyzed to yield isopropylmaleic anhydride. The final product was purified by gas chromotography and its isotopic composition compared with that of the starting material.

All ^{14}C and ^{3}H determinations were made with a Packard Tri-Carb liquid scintillation spectrometer with settings determined by the method of Hendler (1964) and activities calculated according to the procedure of Okita *et al.* (1957).

Results and Discussion

Crystal Structure and Absolute Configuration of Monopotassium α-Isopropylmalate. The space group is $P2_12_12_1$ with cell dimensions a = 6.134 (1), b = 23.433 (5), and c = 6.996 (1) Å, at about 23°. The molecule crystallizes as a hydrate and the measured density is 1.50 g/cm^3, which corresponds to one formula per asymmetric unit.

A total of 1153 reflections was collected to a limiting value of $\sin\theta/\lambda$ = 0.61. The data were empirically corrected for absorption as a function of the angle ϕ, reduced to relative structure amplitudes, and assigned statistical weights. The crystal structure was solved by the heavy-atom method, the initial potassium atomic coordinates having been determined from the Harker sections of a three-dimensional Patterson synthesis. The structure was refined by the block-diagonal approximation of the least-squares method. The quantity $\Sigma w \cdot (|F_o| - |F_c|)^2$ was minimized in these computations (w, F_o, and F_c are, respectively, the weight and observed and calculated structure amplitudes).

Refinement of the anisotropic temperature factors was initiated at R = 0.12. The atomic coordinates of most of the hydrogen atoms were obtained from a difference Fourier synthesis at R = 0.07 and included in the subsequent refinement. It was then possible to extend the refinement to R = 0.048. However, no correction for the $\Delta f''$ components of the potassium and oxygen scattering factors had been made. When these corrections were applied, the structure corresponding to the D$_s$ configuration refined to an R value of 0.040, whereas the one corresponding to the L$_s$ configuration refined to 0.058. In the case of the structure conforming to the D$_s$ configuration the shifts in the parameters were less than one-tenth the computed standard deviations. This implied that further refinement was not warranted by the data. The estimated standard deviations were computed from the sum of the diagonal elements of the inverse matrix of the least-squares normal equations (Table I). The thermal parameters are listed in Table II.

The potassium and oxygen atoms were corrected for anomalous dispersion effects. The values of the dispersion corrections applied in this investigation were, for oxygen, $\Delta f''$ = 0.03 and, for potassium, $\Delta f'$ = 0.37 and $\Delta f''$ = 1.07 (Cromer and Liberman, 1970). The bond distances and absolute con-

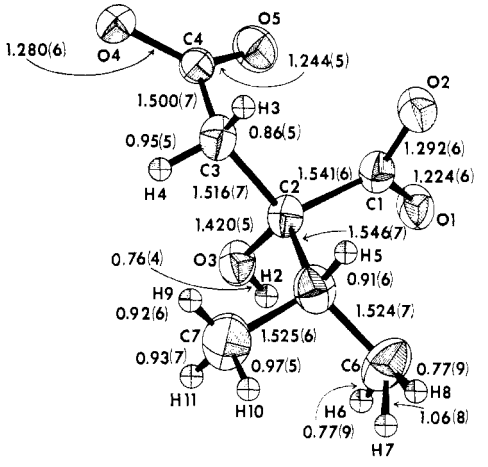


FIGURE 1: Bond distances and absolute configuration of the α-isopropylmalate anion.

TABLE I: Atomic Coordinates and Estimated Standard Deviations for Crystalline Monopotassium α-Isopropylmalate.

Atom	X	Y	Z
K	0.8802 (1)	0.77940 (5)	0.7995 (1)
O(1)	0.6204 (5)	0.6808 (1)	0.8666 (5)
O(2)	0.6256 (5)	0.6032 (1)	0.6824 (5)
O(3)	1.0253 (5)	0.6618 (1)	0.9970 (5)
O(4)	1.3129 (5)	0.6484 (1)	0.5134 (5)
O(5)	0.9828 (6)	0.6832 (1)	0.5641 (5)
C(1)	0.7080 (7)	0.6373 (2)	0.8084 (7)
C(2)	0.9249 (7)	0.6165 (2)	0.8948 (7)
C(3)	1.0842 (7)	0.6005 (2)	0.7381 (7)
C(4)	1.1273 (8)	0.6473 (1)	0.5968 (6)
C(5)	0.8715 (9)	0.5659 (1)	1.0283 (7)
C(6)	0.7028 (10)	0.5813 (2)	1.1795 (10)
C(7)	1.0743 (10)	0.5414 (2)	1.1240 (10)
W	0.8268 (6)	0.7327 (2)	0.2515 (5)
H(2)	0.955 (7)	0.678 (1)	1.066 (6)
H(3)	1.031 (8)	0.572 (1)	0.674 (7)
H(4)	1.217 (9)	0.589 (2)	0.798 (8)
H(5)	0.810 (8)	0.538 (1)	0.951 (7)
H(6)	0.739 (12)	0.613 (3)	1.256 (10)
H(7)	0.718 (13)	0.548 (3)	1.285 (12)
H(8)	0.650 (17)	0.554 (4)	1.221 (14)
H(9)	1.157 (11)	0.520 (2)	1.038 (9)
H(10)	1.031 (9)	0.509 (2)	1.205 (8)
H(11)	1.146 (12)	0.568 (2)	1.197 (10)
H(12)	0.846 (13)	0.768 (3)	0.290 (11)
H(13)	0.862 (9)	0.715 (2)	0.338 (7)

figuration of the molecule are shown in Figure 1; the bond angles are given in Table III.[3] The results of the *R*-factor ratio test were that, on a statistical basis, the L$_S$ configuration was rejected at least at the 0.005 significance level, and it is established that the stereoisomer which is an intermediate in the biosynthesis of leucine is (2*S*)-2-hydroxy-2-isopropylsuccinic acid (D$_S$-α-isopropylmalate). This finding together with the earlier demonstration by Gross *et al.* (1963) that the dehydration product II was dimethyl citraconate, and the determination by Calvo *et al.* (1964) of the absolute configuration of compound III (β-isopropylmalate), indicates that the interconversion involves successive trans elimination and trans addition of the elements of water, resulting in an exchange of the positions of attachment of the hydrogen and hydroxyl groups.

[3] See paragraph at end of paper regarding supplementary material.

TABLE II: Thermal Parameters for Crystalline Monopotassium α-Isopropylmalate.[a]

Atom	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
K	206 (2)	14.6 (2)	177 (2)	0 (1)	0 (5)	0 (1)
O(1)	166 (8)	14.5 (6)	178 (7)	17 (4)	−18 (16)	−22 (3)
O(2)	163 (7)	14.8 (6)	184 (7)	0 (4)	−38 (17)	−22 (3)
O(3)	147 (7)	13.0 (5)	165 (7)	−4 (3)	17 (15)	14 (3)
O(4)	170 (8)	20.6 (7)	135 (6)	−6 (4)	22 (14)	15 (4)
O(5)	228 (10)	16.7 (7)	195 (8)	22 (4)	−22 (16)	18 (4)
C(1)	129 (10)	14.1 (8)	136 (9)	−12 (5)	25 (19)	−5 (5)
C(2)	127 (11)	9.3 (7)	142 (9)	−2 (4)	12 (17)	−4 (4)
C(3)	134 (11)	13.1 (8)	171 (11)	2 (5)	24 (19)	−8 (5)
C(4)	156 (11)	12.9 (8)	113 (8)	−16 (5)	0 (20)	−14 (4)
C(5)	190 (12)	12.0 (8)	174 (10)	−7 (6)	−18 (25)	3 (5)
C(6)	268 (16)	17 (1)	238 (15)	−11 (7)	147 (29)	37 (7)
C(7)	255 (17)	19 (1)	259 (15)	13 (7)	18 (29)	54 (7)
W	277 (11)	14.6 (6)	168 (8)	10 (4)	−67 (15)	−14 (3)
H(2)	1.1 (8)					
H(3)	2 (2)					
H(4)	3 (1)					
H(5)	2 (1)					
H(6)	6 (2)					
H(7)	8 (2)					
H(8)	10 (2)					
H(9)	4 (1)					
H(10)	3 (1)					
H(11)	6 (1)					
H(12)	6 (1)					
H(13)	3 (1)					

[a] The temperature factors for nonhydrogen atoms are of the form $\exp[-(b_{11}h^2 + b_{22}k^2 + b_{33}l^2 + b_{12}hk + b_{13}hl + b_{23}kl)]$. Listed above are $b_{ij} \times 10^4$ for nonhydrogen and β for the hydrogen atoms.

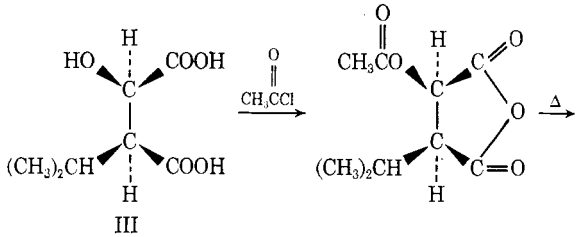
Distribution of Tritium in Doubly Labeled β-Isopropylmalate. OXIDATIVE DEGRADATION TO ISOVALERIC ACID. Final purification of the doubly labeled β-isopropylmalate isolated from culture filtrates was effected by dilution of a suitable aliquot with 100 mg of nonlabeled synthetic racemate A and chromatography on a column of silicic acid (diameter, 2 cm; 20 g of silicic acid; stationary phase, 12.3 ml of 0.05 N H_2SO_4). The column was developed first with 50 ml of chloroform saturated with 0.05 N H_2SO_4. This was followed by chloroform–10% *n*-butyl alcohol saturated with 0.05 N H_2SO_4. Fractions containing the peak for racemate A were combined and evaporated and the product was recrystallized from ether–ligroin. To the recovered compound (total ^3H activity, 430,000 cpm) was added a cold solution of 200 mg of $K_2Cr_2O_7$ in 0.1 ml of concentrated H_2SO_4 and 1.2 ml of water. The reaction mixture was kept in an ice bath and the temperature rose to about 35°. After 4 min, the solution was cooled and extracted five times with ether. The combined ether extracts were dried over anhydrous Na_2SO_4 and evaporated to 1 ml. The ether-extractable acid, determined by titration, was 35% of the theoretical amount; the total ^3H activity was 34,000 cpm. To the

TABLE III: Bond Angles in Crystalline Monopotassium α-Isopropylmalate.

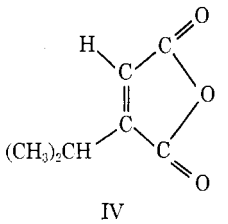
Atoms	Angles (deg)	Atoms	Angles (deg)
O(2)-C(1)-O(1)	124.8 (4)	C(7)-C(5)-C(6)	109.8 (5)
C(2)-C(1)-O(2)	114.2 (4)	C(2)-C(5)-C(7)	112.4 (4)
C(2)-C(1)-O(1)	120.8 (4)	C(7)-C(5)-H(5)	109.4 (3)
C(1)-C(2)-O(5)	107.2 (4)	C(2)-C(5)-H(5)	105.4 (3)
C(5)-C(2)-O(3)	111.3 (4)	H(5)-C(5)-C(6)	107.2 (3)
C(3)-C(2)-O(3)	105.7 (3)	H(6)-C(6)-C(5)	114.8 (4)
C(3)-C(2)-C(5)	112.5 (4)	H(7)-C(6)-C(5)	104.5 (4)
C(1)-C(2)-C(3)	110.6 (4)	H(8)-C(6)-C(5)	110.7 (7)
C(1)-C(2)-O(3)	109.6 (3)	H(6)-C(6)-H(7)	99.1 (6)
C(2)-C(3)-C(4)	114.1 (4)	H(7)-C(6)-H(8)	85.1 (8)
C(2)-C(3)-H(3)	108.9 (3)	H(6)-C(6)-H(8)	122.5 (8)
C(2)-C(3)-H(4)	107.5 (3)	H(9)-C(7)-C(5)	111.4 (4)
H(4)-C(3)-C(4)	109.5 (3)	H(11)-C(7)-C(5)	111.9 (4)
H(3)-C(3)-C(4)	106.6 (3)	H(10)-C(7)-C(5)	108.8 (3)
H(3)-C(3)-H(4)	110.3 (4)	H(9)-C(7)-H(11)	117.2 (6)
C(3)-C(4)-O(4)	118.2 (4)	H(11)-C(7)-H(10)	109.6 (5)
C(3)-C(4)-O(5)	119.4 (4)	H(9)-C(7)-H(10)	96.8 (4)
O(4)-C(4)-O(5)	122.4 (4)	H(2)-O(3)-C(2)	117.9 (3)
C(2)-C(5)-C(6)	112.4 (4)		

ether extract was added 0.07 ml of isovaleric acid as carrier, the mixture was neutralized with dilute NaOH solution, and the product was converted to the *p*-bromophenacyl ester (mp 66°; no depression on admixture with an authentic sample of the *p*-bromophenacyl ester of isovaleric acid). The specific activity of the ester was 9 cpm/mg. Because of the low level of activity of the final product and the possibility of loss by exchange in the process of degradation the data can be interpreted only qualitatively. They suggest that the major portion of the tritium in the starting material was located on the α carbon.

PYROLYSIS OF β-ISOPROPYLMALATE TO ISOPROPYLMALEIC ANHYDRIDE (IV). The pyrolysis mixture, which contained 50 mg of doubly labeled β-isopropylmalate plus 0.4 ml of acetyl chloride, was heated in a sealed tube at 250° for 4 min. The contents of the tube were than diluted with ether and passed in 50-μl quantities through a Beckman GC-2 chromatograph (Calvo *et al.*, 1964), the product emerging at a retention time of 3 min and 45 sec being collected. The total yield was 26 mg. The identity of the product (IV) was established by comparison with the appropriate compound produced on heating teraconic



III



IV

TABLE IV: Isotope Ratios of Compounds Isolated from Cultures of a Leucine Auxotroph of *N. crassa* Incubated with [1-^{14}C]Valine and [^{3}H]Acetate.

Compound	^{3}H (cpm/mg)	^{14}C (cpm/mg)	^{3}H/^{14}C
α-Isopropylmalate (I)	9,860	11,466	0.85
β-Isopropylmalate (III)	10,940	12,520	0.88
Isopropylmaleic anhydride (IV)[a]	8,369	11,640	0.72

[a] Compound IV was obtained by pyrolysis of III.

acid to 180°. This product, which showed a retention time and infrared spectrum identical with IV, yielded, on hydrolysis, isopropylmaleic acid (dimethylcitraconic acid), mp 91°.

The ^{3}H/^{14}C ratios of compounds I, III, and IV are summarized in Table IV. It will be noted that the ratios for the two compounds isolated from the culture medium are virtually identical, while the ratio for isopropylmaleic anhydride is somewhat lower. Again the data can be interpreted only qualitatively. The small apparent loss of tritium on pyrolysis may indicate some exchange of ^{3}H with the medium at the relatively high temperatures employed. Certainly the major part of the tritium is retained and must have been located on the α carbon of III.

Taken together with the data on oxidative degradation, these results suggest that there is a facile enzyme-catalyzed interconversion of α-isopropylmalate and β-isopropylmalate which is stereospecific and which results ultimately in exchange of one specific hydrogen atom with the medium.

It is still possible, however, that this molecular rearrangement occurs by a mechanism involving intramolecular transfer of hydrogen. The two labeled compounds were isolated from culture filtrates. It is not clear how long the compounds were in contact with the isomerase, and for this reason it is impossible to deduce how rapidly the specific hydrogen is exchanged into the medium. *In vitro* experiments employing the purified enzyme will be required to answer this question.

Acknowledgment

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Supplementary Material Available. A listing of observed and calculated structure factor amplitudes will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Deparment, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to document number BIO-73-3346.

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The Potentiating Effect of Adenosine Diphosphate in the Uncoupling of Oxidative Phosphorylation in Potato Mitochondria†

George G. Laties

ABSTRACT: In the absence of added ADP the rate of oxygen utilization by isolated potato mitochondria oxidizing citrate, pyruvate, malate, or succinate is relatively unresponsive to uncouplers of oxidative phosphorylation, *viz.* carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP). *m*-Cl-CCP causes marked respiratory stimulation when added after, or concomitantly with, ADP. The rate of oxygen uptake when *m*-Cl-CCP is added to mitochondria in state 4 is invariably less than the rate typical of state 3. Additional ADP added after *m*-Cl-CCP to state 4 mitochondria elicits the maximal rate of oxygen use characteristic of state 3. A modulator or effector role is imputed to ADP, distinct from its acceptor function in oxidative phosphorylation. ADP potentiation of uncoupler action is second order, with an $s_{0.5}$ of approximately 80 μM. The effector function of ADP is not fulfilled by guanosine nucleotides, nor by AMP or ATP.

Whether oxidative phosphorylation is effected by chemical coupling (see Lehninger, 1970) or by charge separation as per the chemoosmotic theory (Mitchell, 1966), conventional uncouplers are considered to act previous to any involvement of ADP. In fact a classical uncoupler, DnpOH,[1] inhibits not only the P_i–ATP exchange reaction, but also inhibits oxygen exchange between phosphate and water (Cohn, 1953; see Lehninger, 1970), further emphasizing that conventional uncouplers act well before the step in which ADP participates in ATP formation. Nevertheless, with potato mitochondria we have consistently failed to elicit marked respiratory stimulation with uncouplers in the absence of added ADP. By contrast, the respiration of potato mitochondria in state 4, *i.e.*, where added ADP has been largely converted to ATP (Chance and Williams, 1956), is sharply enhanced by uncouplers, and the stimulated respiration is resistant to oligomycin.

The foregoing observations suggest a dual role for ADP in potato mitochondrial electron transport: (1) as a conventional phosphate acceptor in oxidative phosphorylation and (2) as a modulator in the implementation of uncoupler activity. A dual role was suggested some time ago (Laties, 1953), but since the observations leading to the deduction centered largely on α-ketoglutarate oxidation, and coincided with the discovery of substrate-level phosphorylation, the need for ADP to implement uncoupler effectiveness was subsequently erroneously attributed solely to its role in substrate-linked phosphorylation. In this vein, two subsequent examples of the dependence of uncoupler effectiveness on ADP both dealt with pyruvate oxidation—by horsefly sarcosomes in one instance (Van den Bergh, 1964) and by insect mitochondria in the other (Gregg *et al.*, 1964). In each case pyruvate oxidation was complete, and the dependence on ADP was shown to be due to the substrate-level phosphorylation attending α-ketoglutarate oxidation. However, the experiments set out below demonstrate the dual role of ADP with a variety of substrates where substrate-level phosphorylation is not at issue.

Isolated plant mitochondria frequently show an attenuated state 3 on the first presentation of ADP. With several cycles of alternating state 3–state 4, state 3 rises to a maximum, the phenomenon having been called "conditioning" (Raison *et al.*, 1973a). Conditioning apparently characterizes isolated mammalian mitochondria as well (Raison and Lyons, 1971; Raison *et al.*, 1973a). We have subsequently discovered that state 4 brings about conditioning as effectively as alternating cycles, and that in fact continuous state 3 is inimical to maximal conditioning (Raison *et al.*, 1973b). Nevertheless, conditioning is abetted by ADP in the absence of oxidative phosphorylation, a fact which can be demonstrated by incubating mitochondria with ADP in the absence of inorganic phosphate, and subsequently initiating state 3 by addition of the latter. ADP is consequently implicated in conditioning, and apparently for reasons other than its role as phosphate acceptor in oxidative phosphorylation. Thus we must ask

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[1] Abbreviations used are: DnpOH, 2,4-dinitrophenol; Mercap, 2-mercaptobenzothiazole, Na salt; *m*-Cl-CCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.