however, a small but definite amount of enzymatic activity even when special precautions were taken to eliminate DPGA from the reaction. This activity was ascribed to an activated form of the enzyme, probably a phosphorylated form. Another possibility was that cofactor DPGA was tightly bound to the enzyme and was therefore always present. In order to differentiate between these two alternatives, use has been made of C^{14}- and P^{32}-labeled substrates.

Carboxyl-labeled 3-phosphoglyceric acid (3PGA) was obtained by treating ribulose 1,5-diphosphate with C^{14}-sodium bicarbonate in the presence of purified carboxydismutase as described by Calvin, *et al.*[2,3] To a solution containing 0.17 μmole of C^{14}-3PGA, 0.15 μmole DPGA and 50 μmoles of imidazole-HCl buffer *p*H 7.0 was added 0.2 μmole of crystalline enzyme (final volume 1.5 ml.). After two hours of incubation at 30° the solution was dialyzed extensively against several changes of distilled water and sodium chloride solution. The final dialysate possessed no radioactivity but the protein solution was radioactive, and the amount of radioactivity in this solution indicated that there was about one mole of phosphoglyceric acid per 100 moles of protein. It appears, therefore, that although substrate is tightly bound to the enzyme, not enough is present to account for all the activity in the reaction mixtures where DPGA is excluded.

Phosphate-labeled DPGA was isolated from fluoride-poisoned fermentation mixtures by barium precipitation,[4] and was purified by paper chromatography with a 2-propanol/NH_4OH/water (70:10:20) solvent system. To a solution containing 50 μmoles of DPGA and 200 μmoles of imidazole–HCl buffer *p*H 7.0 was added 0.2 μmole of crystalline enzyme (final volume 5.6 ml.). After incubation and dialysis similar to that described previously, the protein solution was evaporated to dryness. It contained bound P^{32}, which was shown to be linked to the protein: Partial hydrolysis of the protein was carried out by dissolving it in 11 *N* hydrochloric acid and incubating at 40° for 72 hours. The hydrolysate, after removal of the hydrochloric acid, was passed through a Dowex 50 (H^+) resin column as described by Flavin.[5] The eluate was rechromatographed on paper with the 2-propanol solvent previously described and the region containing the P^{32}-labeled peptide material was eluted. This material after hydrolysis at 110° in a sealed tube with 5.7 *N* hydrochloric acid for 20 hours was chromatographed in the phenol–water system of Block.[6] Development of the chromatogram with ninhydrin indicated that the amino acids serine, alanine, and glutamic acid were present along with P^{32}-inorganic phosphate. Presumably in the peptide material resulting from partial hydrolysis the phosphate is esterified to the serine hydroxyl.

Determination of the P^{32} in the phosphorylated peptide fraction purified by chromatography indicated that 94% of the protein molecules had been phosphorylated. It appears that sufficient phosphate is present on the enzyme to account for the observed activity in the absence of DPGA, and that phosphoglyceric acid mutase has a mechanism of action similar to that of phosphoglucomutase.[7]

(2) J. Mayaudon, A. A. Benson and M. Calvin, *Biochim. Biophys. Acta*, **23**, 342 (1957).

(3) The C^{14}-3PGA was prepared by Dr. N. Pon and the author gratefully acknowledges the gift of this compound.

(4) C. Neuberg and H. Lustig, *Arch. Biochem.*, **1**, 311 (1942).

(5) M. Flavin, *J. Biol. Chem.*, **210**, 771 (1954).

(6) R. J. Block, *Anal. Chem.*, **22**, 1327 (1950).

(7) V. A. Najjar and M. E. Pullman, *Science*, **119**, 631 (1954). E. P. Kennedy and D. E. Koshland, *J. Biol. Chem.*, **228**, 419 (1957).

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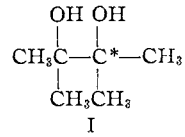
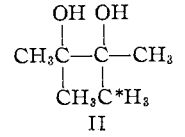
THE CARBON ISOTOPE EFFECT IN THE PINACOL-PINACOLONE REARRANGEMENT: A REINVESTIGATION

Sir:

Intermolecular carbon-isotope effects in the acid-catalyzed rearrangements, above 80°, of 2,3-dimethyl-2,3-butanediol-2-C^{14} [I] and 2,3-dimethyl-



I



II

2,3-butanediol-2-*methyl*-C^{14} [II] have been reported recently.[1] The magnitude of these reported isotope effects [k^*/k for I, 0.74; k^*/k for II, 0.48] is astonishing, particularly in the case of II, in which the isotopic bond should never be *directly* involved in the initial bond-breaking process. In addition the data of Duncan and Lynn, in two instances,[1b] fail to give a material balance. We have therefore reinvestigated the intermolecular isotope effects in the acid-catalyzed rearrangements of I and II.[1c] We find that in the presence of 0.295 *N* hydrochloric acid at 100°, within the limits of our experimental error, I exhibits an intermolecular isotope effect no greater than 3% [$k^*/k = 0.97$] and II exhibits no isotope effect at all [$k^*/k = 1.00$].

Acetic-1-C^{14} acid was converted to acetone-*carbonyl*-C^{14} by passage over manganous carbonate at 420–450°[2] [acetone-2,4-dinitrophenylhydrazone, m.p. 125–126°, radioactivity assay, 2.239 ± 0.001 mc./mole]. Isotope position isomer I (hexahydrate, m.p. 45.5–47°) was prepared from the foregoing acetone.[3] Periodate[1] cleavage of I produced acetone-*carbonyl*-C^{14} whose 2,4-dinitrophenylhydrazone had a m.p. of 125–126° and a radioactivity assay of 2.215 ± 0.005 mc./mole.

(1) a) J. F. Duncan and K. R. Lynn, *Australian Journal of Chemistry*, **10**, 7–25 (1957); (b) **10**, 18 (1957), Table II, line 3; (c) We are pleased to acknowledge a discussion with Professor J. F. Duncan in May of 1957, during which he brought his results to our attention. During this discussion (as in ref. 1a), Professor Duncan indicated that the isotope effect values calculated from his data may "be apparent, rather than real." Recently (personal communication), Professor Duncan has stated that some of his original results with K. R. Lynn may possibly be explainable as a consequence of a keto-enol equilibrium which prevents complete precipitation, at low temperature, of the 2,4-dinitrophenylhydrazone of pinacolone. If the pinacolone added as carrier were all in the keto-form, then an apparent but unreal isotope effect would be observed.

(2) W. M. Cumming, I. V. Hopper and T. S. Wheeler, "Systematic Organic Chemistry," 4th Ed., Constable and Co., Ltd., London, 1950, p. 101.

(3) "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 459.

Compound I (3.00 g., hexahydrate) was allowed to react for 30 minutes with 400 ml. of 0.295 *N* hydrochloric acid at 100 ± 0.5°, and the reaction was then quenched in ice-water containing sodium bicarbonate. The organic material was isolated by continuous extraction with ether, and the unreacted pinacol (1.2 g.) was isolated from the ether solution as the hexahydrate, after addition of a minimum quantity of water. Periodate[1] cleavage of the recovered pinacol afforded acetone-*carbonyl*-C^{14} whose 2,4-dinitrophenylhydrazone (m.p. 125–126°) had a radioactivity assay of 2.240 ± 0.004 mc./mole. The pinacolone remaining in the foregoing ether solution was converted to the 2,4-dinitrophenylhydrazone, m.p. 126–127° (depression when mixed with acetone-2,4-dinitrophenylhydrazone!), radioactivity assay, 4.388 ± 0.012 mc. mole.

Compound II (hexahydrate, m.p. 45–46.5°) was prepared from redistilled biacetyl (b.p. 83–84°) and methyl-C^{14}-magnesium iodide. A repetition of the experiment described above (by which I was subjected to rearrangement) led to the reisolation of 2.1 g. of II-hexahydrate. Cleavage of reisolated II by periodate produced acetone-*methyl*-C^{14} whose 2,4-dinitrophenylhydrazone (m.p. 125–126°) had a radioactivity assay of 2.062 ± 0.001 mc./mole. The pinacolone 2,4-dinitrophenylhydrazone isolated from the same reaction mixture had a m.p. of 126–127°, and a radioactivity assay of 4.122 ± 0.017 mc./mole. From the kinetic data of Duncan and Lynn[4] the conditions for both of the foregoing experiments correspond to values of $a/(a - x)$ in the first-order rate equation of 1.36, or a value of f (fraction reacted) of 0.264. From these data, therefore, it can be calculated[5] that the isotope effects (k^*/k) are, within experimental error of ±1%, for the rearrangement of I, equal to or less than 0.97, for the rearrangement of II, unity.[6]

(4) J. F. Duncan and K. R. Lynn, *J. Chem. Soc.*, 3513 (1956), Table 2.

(5) W. Stevens and R. Attree, *Can. J. Research*, **B27**, 807 (1949); J. Ying-Peh Tong and P. E. Yankwich, *J. Phys. Chem.*, **61**, 540 (1957).

(6) This paper is based upon work performed at Oak Ridge National Laboratory which is operated by Union Carbide Corporation for the Atomic Energy Commission.

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RECEIVED JULY 7, 1958

THE ASSOCIATION BEHAVIOR OF β-LACTOGLOBULINS A AND B

Sir:

The discovery of Aschaffenburg and Drewry that β-lactoglobulin consists of two genetically different proteins[1] has led to a reexamination of its electrophoretic heterogeneity.[2,3,4,5,6] Ogston and Tombs[7] found that at *p*H 4.65 β-lactoglobulin A[1] resolves into two peaks on the descending side while the B protein shows only a pronounced skewness of the boundary. From this they concluded that β-lactoglobulin A is primarily responsible for the aggregation between *p*H 3.7 to 5.2,[5,8] while β-lactoglobulin B aggregates to a considerably lesser extent.

Klostergaard and Pasternak[9] reported electrophoretic patterns identical with those of Ogston and Tombs,[7] and also some ultracentrifugal data, with the opposite conclusion that only β-lactoglobulin B associates.

In the course of studies on the molecular behavior of β-lactoglobulin between *p*H 1.5 and 5.5[8,10] we have examined for evidence of aggregation eight samples of β-lactoglobulin A and twelve of β-lactoglobulin B prepared in our laboratory from the milk of individual cows as well as samples of the two proteins kindly given to us by Dr. R. Aschaffenburg. The results obtained showed that all samples of β-lactoglobulin A aggregate strongly at *p*H 4.65 and 2° while none of the samples of β-lactoglobulin B do.

A correlation of the ultracentrifugal and electrophoretic patterns is given in Fig. 1. The ultracentrifugal patterns of the β-lactoglobulin prepared from pooled milk (designated as "normal") and of β-lactoglobulin A exhibits two peaks with $s_{20,w}$ values at 2% protein of 2.8 and 5.3 *S*, corresponding to monomer and aggregate, respectively, while β lactoglobulin B gives a single peak with $s_{20,w}$ of 2.7 *S* for 2% protein. Increasing the protein concentration up to 7% resulted in no evidence of aggregation. The electrophoretic patterns are

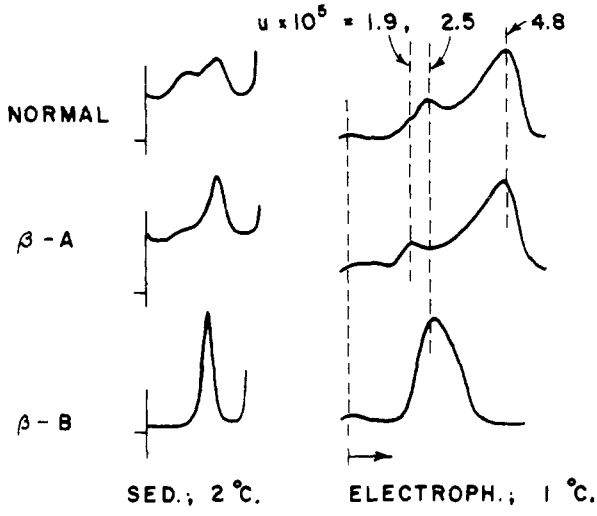

Fig. 1.—Tracings of ultracentrifugal and electrophoretic patterns (descending) of various β-lactoglobulins in *p*H 4.65 acetate buffer, Γ/2 = 0.1. Both sedimentation and electrophoretic migration proceed from left to right: sedimentation, 59,780 r.p.m.; "Normal" and β-A, 1.4% protein, 160 min.; β-B, 7% protein, 352 min.; electrophoresis, 1.6% protein, 8,000 sec. at 9.7 volts/cm.

(1) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955); **180**, 376 (1957).

(2) C. H. Li, THIS JOURNAL, **68**, 2746 (1946).

(3) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(4) B. D. Polis, H. W. Schmukler, J. H. Custer and T. L. McMeekin, THIS JOURNAL, **72**, 4965 (1950).

(5) A. G. Ogston and J. M. A. Tilley, *Biochem. J.*, **59**, 644 (1955).

(6) S. N. Timasheff, unpublished experiments.

(7) A. G. Ogston and M. P. Tombs, *Biochem. J.*, **66**, 399 (1957).

(8) R. Townend and S. N. Timasheff, *Arch. Biochem. Biophys.*, **63**, 482 (1956).

(9) H. Klostergaard and R. A. Pasternak, THIS JOURNAL, **79**, 5671 (1957).

(10) R. Townend and S. N. Timasheff, *ibid.*, **79**, 3613 (1957).