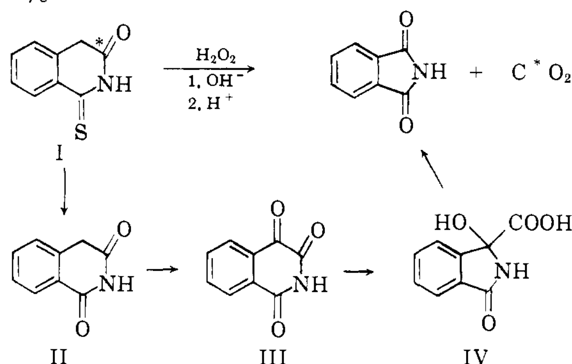
novel, one-step, ring-shrinking process, in which the benzilic acid rearrangement appears to constitute a phase, which has the effect of converting 1,3(2,4)-isoquinolinediones (II) (homophthalimides) or their monothio analogs (I) to phthalimides. The change is accomplished by treatment with excess hydrogen peroxide and aqueous alkali at room temperature or slightly above for periods of less than three hours, then acidification and brief warming. We have effected it with seven examples (2a-thiohomophthalimide, 4-methyl-, 4-chloro-, 5-methyl-, 5-methoxy-, and 5,6-benzo-thiohomophthalimides, and N-methylhomophthalimide) in yields of 46 to 81%.



The reaction with monothiohomophthalimides, with which most of our experiments were carried out, proceeds by initial oxidative desulfurization to homophthalimides, which can be isolated in good yield if the amount of peroxide is limited to four molar equivalents. Oxidation to phthalonimides (III) through epoxidation of the enol double bond presumably follows, but such compounds cannot be isolated, for they are too rapidly altered in alkaline solution[4] (oxidation in acid solution gives isolable phthalonimide, however[4]). If the peroxide is destroyed before acidification, a substance is formed which agrees with the described[4] alteration product of phthalonimide (largely 3-hydroxyphthalimidine-3-carboxylic acid (IV), "phthalonamic acid"[4]), but not phthalimide. If the reaction mixtures (containing excess peroxide) are kept cold during acidification, phthalimide is not formed and carbon dioxide is not evolved until the mixture subsequently is warmed gently. Phthalonimide prepared independently gives the same products as homophthalimide under similar conditions.

Although the over-all reaction in effect deletes a methylene group, it is the carbonyl group of I that is eliminated. When the reaction was carried out with thiohomophthalimide prepared[5] from phenylacetic acid 1-C^{14}, nearly all the radioactivity appeared in the evolved carbon dioxide. The carbon dioxide obtained by the Hofmann rearrangement of the resulting phthalimide had activity only just detectably above normal background. The ring-shrinking step is thus a rearrangement of the benzilic acid type, in which the bond-breaking and bond-forming steps occur at a nitrogen atom. Instances of benzilic acid rearrangement in a nitrogenous heterocyclic system have been reported before,[3] but we believe this is the first case where migration of nitrogen has been established.

(4) S. Gabriel and J. Colman, *Ber.*, **33**, 996 (1900).

(5) P. A. S. Smith and R. O. Kan, *J. Am. Chem. Soc.*, **82**, 4753 (1960).

In view of the possibility that the ring-shrinking step occurs through a phthalonimide anion and an intermediate three-membered ring, analogous to the hetero Favorskii rearrangement proposed by Sarel and Greenberger,[6] we carried out the reaction with 2-methyl-1,3(2,4)-isoquinolinedione (N-methylhomophthalimide), also labelled at the 3-position (the "aliphatic" carbonyl). The same over-all conversion occurred readily, and N-methylphthalimide was obtained in good yield. However, the evolved carbon dioxide had only 85% of the radioactivity to be expected if it had been derived solely from the 3-carbonyl, and a significant amount of isotope was detected in the carbon dioxide derived from the resulting N-methylphthalimide carbonyls by conversion to phthalimide followed by Hofmann rearrangement. Thus even when phthalonimide anion cannot be formed, migration by nitrogen predominates, although aryl migration becomes a minor competing process. Phthalamic acids are not intermediates, for they are not converted to phthalimide under the reaction conditions; hydrolytic ring opening to a phthalonamic acid followed by reclosure at the ketonic carbonyl is not ruled out (nor is the analogous scheme for the rearrangement of alloxan), although there are reasons to doubt it. A mechanism analogous to one proposed for the rearrangement of alloxan at high pH by Kwart and Sarasohn[1] conforms best to the facts, and our results provide strong support for their proposal.

Because of its simplicity, mildness, and satisfactory yields, we believe this reaction has useful application in synthesis as well as structure proof. It already has enabled us to identify as 2a-thiohomo-1,2-naphthalimide (5,6-benzo-1-thio-1,3(2,4)-isoquinolinedione) the compound earlier believed[5] to be its isomer, 8a-thio-1-homo-1,8-naphthalimide; both give by simple hydrolysis decarboxylic acids of the same melting point, but the imides of one less carbon are strongly differentiated. Furthermore, by a combination of the present reaction with the recently reported synthesis of thiohomophthalimides,[5] some phthalimides can be synthesized that were previously available only by circuitous routes.

(6) S. Sarel and A. Greenberger, *J. Org. Chem.*, **23**, 330 (1958).

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EVIDENCE FOR MALIC SYNTHETASE IN ANIMAL TISSUES

Sir:

Malic synthetase, one of the key enzymes of the glyoxylate cycle,[1] was demonstrated both in microorganisms[2,3] and in plants.[4,5] Recent report by Madsen[6] indicated that the key enzymes of the

(1) H. L. Kornberg and H. A. Krebs, *Nature*, **179**, 988 (1957).

(2) D. T. O. Wong and S. J. Ajl, *J. Am. Chem. Soc.*, **78**, 3230 (1956).

(3) H. L. Kornberg, *Biochem. J.*, **68**, 549 (1958).

(4) H. L. Kornberg and H. Beevers, *Biochim. Biophys. Acta*, **26**, 531 (1957).

(5) C. Bradbeer and P. K. Stumpf, *J. Biol. Chem.*, **234**, 498 (1959).

(6) N. B. Madsen, *Biochim. Biophys. Acta*, **27**, 199 (1958).

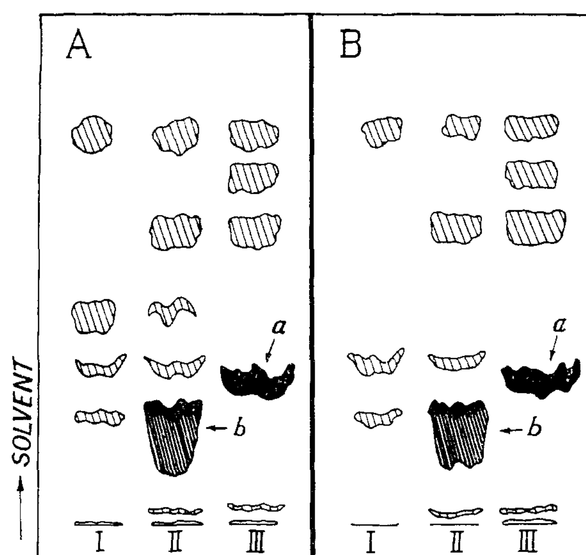

Fig. 1.—Radioautograms of two typical paper chromatograms showing the formation of radioactive malate from (A) acetate-2-C^{14} and (B) acetate-1-C^{14}: I, incubation with acetate-C^{14}: II, acetate-C^{14} + cofactors: III, acetate-C^{14} + cofactors + glyoxylate; solvent was 1-butanol-acetic acid-water (4:1:1), as developing solvent by ascending technique unidimensionally. Malate and malic acid have different R_f values in this solvent. The radioactive spot (position a, system III) corresponded to malate. This spot when eluted and treated with mineral acid and rechromatographed in the same solvent, corresponded to malic acid. Radioautography was carried out on X-ray films (Ilford) for a period of 7 days: position a, *i.e.*, malate only appeared in incubation system III where glyoxylate was present; position b = acetylCoA,[7] only present in II where acetate was incubated with CoA and ATP and was utilized in III by its reaction with glyoxylate and thereby forming malate. In system III, apart from malate, other substances with relatively low activities also had appeared. The degree of cross-hatching indicates the approximate radioactivity of a spot.

glyoxylate cycle do not occur in the animal tissues. It is now well established that the glyoxylate cycle is mainly responsible for a net conversion of fat to carbohydrate.[1,4,5] Under the present evidence, it has become difficult to explain the mechanism involved where the conversion of fat to carbohydrate in animals is known to occur.[9] For this reason we thought it necessary to test once more for the presence of malic synthetase and isocitratase in animal tissues. This report is an account of such experiments giving the evidence in favor of the occurrence of malic synthetase in different tissues from rat, guinea pig and rabbit. The experiments reported here were designed in some modified way from that of Madsen,[6] which enabled us to demonstrate malic synthetase activity in animal tissues.

Normal rat liver tissue was homogenized in an equal volume of 0.1 M phosphate buffer of pH 7.0 and centrifuged at 20,000 × g for 30 minutes. The supernatant then was used as enzyme source. This crude preparation was found to have malic synthetase activity. The incubation was carried out at 37° for a period of one hour in 0.5 ml. volume containing 50 μM. phosphate buffer, pH 7.0, 10 μc. acetate-1-C^{14} or acetate-2-C^{14}, 1 μM. inactive acetate, 0.1 μM. CoA, 1 μM. ATP, 1 μM. reduced glutathione, 2 μM. $MgCl_2$ and 4 μM. glyoxylate along with the enzyme preparation (0.2 ml.). The reaction was stopped with 75% alcohol. The supernatant after centrifugation was evaporated to dryness under vacuum. The dried material then was dissolved in water and the water extract of the reaction mixture was subjected to paper chromatography in 1-butanol:acetic acid:water (4:1:1) solvent unidimensionally. The radioactive components were detected by radioautography on X-ray films. Synthesis of radioactive malate took place when glyoxylate was present in the system along with acetate and other cofactors (Table I). Glyoxylate could not be replaced by any other Krebs cycle intermediates. Malonate did not inhibit this synthesis. Reaction was completely dependent on CoA and ATP. A typical radioautogram is shown in Fig. 1, which indicates the formation of malate (experiment III) where glyoxylate was added in the system. That the new reaction product was malate was proved by several facts: (i) it gave the same R_f value as that of authentic malate in four different solvents and was identified by cochromatography, (ii) the specific activity of the new radioactive spot was found to diminish when inactive malate was added in the incubation mixture, (iii) by the fluorometric assay method of Hummell,[10] (iv) radioactivity of the new spot was found to diminish when either DPN^+ or TPN^+ was added in the complete incubation mixture, since the enzyme preparation also had malic dehydrogenase and malic enzyme activities which probably had utilized the formed malate. Malic synthetase also was found to be present in different tissues (liver, kidney) from rat, guinea pig and rabbit. It was absent in heart and brain tissues of all the three animals tested.

TABLE I

	Radioactivity in malate,[b] c.p.m.
Complete system[a]	1572
Minus CoA	140
Minus ATP	41
Minus GSH	685
Minus $MgCl_2$	1172
Minus glyoxylate	50

[a] Radioactive acetate used was labeled either in 1 or 2 position. [b] Separated by paper chromatography in 1-butanol–acetic acid–water and counted directly on the chromatogram, position a in Fig. 1. The relatively low synthesis of malate probably resulted from the facts that: (i) reaction was studied with acetate-C^{14} and not with acetylCoA-C^{14}, which is the actual substrate for malic synthetase, and probably the synthesis of acetylCoA was rate limiting, (ii) synthesized malate had chances for its utilization by both malic dehydrogenase and malic enzyme which also were present in the enzyme preparation, (iii) added glyoxylate had also other metabolic pathways such as its reduction to glycolate by the glyoxylic reductase activity of the tissue preparation and lastly (iv) acetate-C^{14} was considerably oxidized to $C^{14}O_2$ (18–20%) indicating the presence of tricarboxylic acid cycle enzymes. Almost a similar rate of incorporation also was observed by others[4] where they had a similar situation regarding the presence of other enzymes in their malic synthetase preparation.

(7) AcetylCoA was identified by eluting the new radioactive spot from system II and performing a hydroxymate reaction,[8] which was found positive.

(8) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

(9) E. O. Weinman, E. H. Strisower and I. L. Chaikoff, *Physiol. Rev.*, **37**, 252 (1957).

(10) J. P. Hummell, *J. Biol. Chem.*, **180**, 1225 (1949).

Experiments also were carried out to detect the presence of isocitratase in the rat liver preparation. The assay procedure adopted was that of Kornberg and Beevers.[4] Up till now it has not been possible to detect this enzyme activity by the assay procedure adopted, perhaps because the supernatant preparation which had both isocitric dehydrogenase and glyoxylic acid reductase activities may have caused the removal of both isocitrate and glyoxylate. A similar situation also was faced by others[11] while working with castor bean preparations. Further work in this line is in progress with some modified system for the test of isocitratase activity, and a full account will be published elsewhere.

Acknowledgment.—We are indebted to Dr. B. C. Guha of this department for his kind interest and advice during the work. Grateful thanks are also due to Dr. P. K. Stumpf of the University of California, Davis, California, for some valuable suggestions. We are also indebted to Dr. I. Zelitch of New Haven, Connecticut, for a gift sample of glyoxylate and to Sigma Chemical Company, U.S.A., for samples of glyoxylate and CoA.

(11) A. Marcus and J. Velasco, *J. Biol. Chem.*, **235**, 563 (1960).

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THE OSMOTIC APPROACH TO THE PHENOMENON OF ELECTROLYTE INVASION OF ION-EXCHANGE RESINS

Sir:

The water activity of ion-exchange resins which have been invaded by non-exchange electrolyte has been measured by an isopiestic technique. Preliminary results have been obtained for the systems LiCl–LiR and NaCl–NaR where the symbol R represents Dowex-50 X-8. The resin samples were first equilibrated with external salt solutions of various concentrations to obtain a range of mole fractions of electrolyte within the resin phase. The invaded resin samples after separation from the aqueous salt solutions by centrifugation through a sintered glass disc were placed in test-tubes and allowed to equilibrate with saturated salt solutions which ranged in water activity from 0.9248 to 0.0703, so as to obtain different water contents and thus different molalities at the same electrolyte mole fraction. This method does not appear to have been used previously. The time needed to reach equilibrium varies from a few days to 3–4 months. No effort was made to hasten the approach to equilibrium either by use of silver crucibles and copper blocks to enhance thermal conductivity, by evacuation of the system or by circulation of the water vapor. The ambient temperature of 24 ± 2° was employed.

Thus, one obtains the osmotic coefficient, ϕ, as a function of the total molality for several different mole fractions of salt.

$$\log a_w = -0.00782\phi(2m_{MCl} + m_{MR}) + \pi V_{H_2O}/2.303RT$$

where a_w = external water activity, m = internal molality, π = swelling pressure, V_{H_2O} = partial molal volume of water in the resin phase. The swelling pressure, π, is calculated from the empirical equation of Glueckauf,[1] $\pi = 1.23(V_e - 192)$, for an 8% DVB resin where V_e is the equivalent volume of the swollen invaded resin which is obtained by a direct pycnometric density measurement and the numerical constants represent the modulus of elasticity and the unswollen or matrix resin volume, respectively.

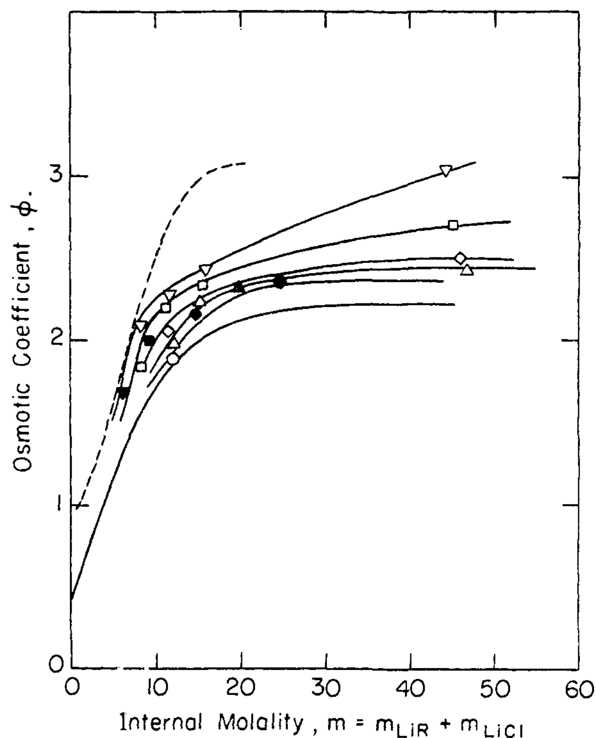

Fig. 1.—Osmotic coefficient as a function of the internal molality, $m = m_{LiR} + m_{LiCl}$: pure LiR ——, pure LiCl ----, mole fraction LiCl 0.351 ○; 0.303 △; 0.282 ◇; 0.217 □; 0.100 ▽. Shaded symbols refer to separated resins samples without additional isopiestic equilibration.

The results for the system LiCl–LiR are presented in Fig. 1 as a plot of the osmotic coefficient, ϕ, *vs.* the internal molality, $m = m_{LiCl} + m_{LiR}$. The solid points represent values obtained from the separated resin samples without additional isopiestic equilibration. Also included for comparison are the curves for pure $^1/_2$% D.V.B. LiR obtained by Soldano[2] and by Glueckauf[1] for molalities above 15, and for pure LiCl. The variation of ϕ with composition is not as expected; the curve for a LiCl mole fraction of 0.100 lies closer to that for pure LiCl than does the curve for a LiCl mole fraction of 0.351. The curves for the mixed electrolyte systems could be fitted to an equation of the type used by Soldano for pure LiR

$$\phi = \frac{am}{1 + bm} + \phi_0$$

where a, b and ϕ_0 are constants, since the curves have somewhat similar shapes. If a, b, and ϕ_0 are smoothly varying functions of the composition, these results together with the LiCl activity of the

(1) E. Glueckauf, *Proc. Roy. Soc.* (*London*), **A214**, 207 (1952).

(2) B. Soldano and Q. V. Larson, *J. Am. Chem. Soc.*, **77**, 1331 (1955).