soma equiperdum in mice as compared with the aminonucleoside from puromycin rated 1: **isobutylamino**, 2; **(methyl)-propylamino**, 4; **diethylamino**, 4–8; **(ethyl)-propylamino**, 2; **dipropylamino**, 4–8; **diallylamino**, 1; **(butyl)-ethylamino**, 1; **cyclohexylamino**, < 0.5; **1-piperidyl**, 0.25; **benzylamino**, < 0.25; **furfurylamino**, 0.5.

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RECEIVED JULY 3, 1956

A POSSIBLE ENZYMATIC ROLE OF ERGOTHIONEINE[1]

Sir:

Previous reports from this laboratory have shown the existence of essentially two types of enzymes which split DPN at the nicotinamide–ribose linkage. One type is nicotinamide sensitive, or inhibited by nicotinamide, such as the DPNases from beef spleen[2] and pig brain,[3,4] the second type is relatively unaffected by the presence of nicotinamide as exemplified by the *Neurospora* DPNase.[5]

We have recently described[6] a nicotinamide-sensitive enzyme from human and bovine erythrocytes which splits nicotinamide riboside (NR). Partial purification of this enzyme, from hemolysates, has been effected by fractional ammonium sulfate precipitation. Purification of the nicotinamide ribosidase resulted in retention of the splitting activity of NR with complete loss of the nicotinamide sensitivity. However, the nicotinamide sensitivity could be restored to the partially purified enzyme by reconstitution with a 5% trichloroacetic acid extract of the intact washed erythrocytes, or by the supernatant fraction resulting from the ammonium sulfate precipitation of the enzyme.

The same factor from the erythrocytes could also impart nicotinamide sensitivity to the *Neurospora* DPNase. This DPNase does not promote an exchange reaction of C^{14}-nicotinamide for the nicotinamide moiety of DPN. However the addition of the factor can endow the *Neurospora* enzyme with exchange properties. Evidence has also been obtained that the nicotinamide ribosidase from erythrocytes will promote an exchange reaction only in the presence of the factor.

Preliminary chemical studies revealed that the factor contained a sulfhydryl grouping which was inactivated by bromine oxidation, or by metal ions. Enzymatic activity of the oxidized product was not restored by the addition of reducing agents. Inactivation by metal ions, however, did respond to reactivation by either glutathione, cysteine or ascorbic acid.

That the factor showed sulfhydryl properties led us to try a number of known sulfur compounds for activity. The only compound which showed activity in promoting nicotinamide sensitivity to the nicotinamide ribosidase and the *Neurospora* DPNase was ergothioneine (the betaine of 2-thiolhistidine). The concentrations of authentic ergothioneine (1×10^{-5} *M*) utilized routinely for demonstrating nicotinamide sensitivity, associated with the *Neurospora* DPNase and erythrocyte nicotinamide ribosidase, were of the same order of magnitude as that contained in the trichloroacetic acid extract. Glutathione, cysteine, coenzyme A or 2-thiolhistidine were without effect.

Authentic ergothioneine (Mann Research Laboratories, Inc.) and the factor from washed erythrocytes behaved similarly when chromatographed on an anionotropic aluminum oxide (M. Woelm Eschwege, Alupharm Chemical) column eluted with an ethanol–formic acid mixture according to the directions of Melville, Horner and Lubschez.[7] The resolution of the biologically active material, from the trichloroacetic acid extract as seen in Fig. 1, was coincident with material reacting positively to the Hunter test,[8] as modified by Melville and Lubschez,[9] for ergothioneine and 2-thiolhistidine.

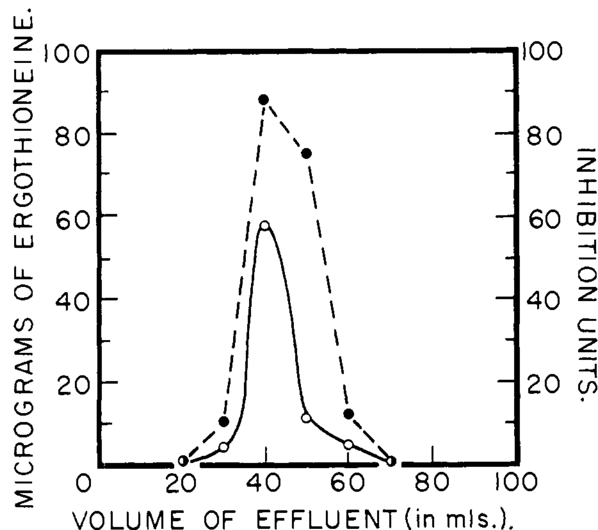

Fig. 1.—Partition chromatography of the trichloroacetic acid extract of human erythrocytes: inhibition units represent the per cent. inhibition of nicotinamide ribosidase in the presence of 10^{-2} *M* nicotinamide and a 0.1-ml. sample from each 5-ml. fraction collected. All enzymatic reaction mixtures contained 3 units of nicotinamide ribosidase (1 unit is that quantity of enzyme which will split 0.1 micromole of NR in 6 minutes), 10^{-5} *M* glutathione, 0.1 *M* phosphate buffer (*p*H 7.4) and water to a final volume of 0.7 ml. A preincubation period of 5 minutes was followed by the addition of 1.4 micromoles of NR. The dashed line represents the inhibition units whereas the solid line depicts the ergothioneine concentration.

(1) Research contribution of the McCollum-Pratt Institute, aided by grants from the National Cancer Institute of the National Institutes of Health (Grant No. C-2374C), and from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

(2) L. J. Zatman, N. O. Kaplan and S. P. Colowick, *J. Biol. Chem.*, **200**, 197 (1953).

(3) L. J. Zatman, N. O. Kaplan, S. P. Colowick and M. M. Ciotti, *ibid.*, **209**, 453 (1954).

(4) N. O. Kaplan and M. M. Ciotti, *ibid.*, in press.

(5) N. O. Kaplan, S. P. Colowick and A. Nason, *ibid.*, **191**, 473 (1951).

(6) L. Grossman, *Fed. Proc.*, **15**, 266 (1956).

(7) D. B. Melville, W. H. Horner and R. Lubschez, *J. Biol. Chem.*, **206**, 221 (1954).

(8) G. Hunter, *Canad. J. Res.*, Sec. E, **27**, 230 (1949).

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On the basis of these results we have tentatively identified the factor as ergothioneine or some closely related compound.

Details of the mechanism of the enzyme-catalyzed exchange reaction and the significance of ergothioneine will be discussed in subsequent papers.

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RECEIVED JUNE 13, 1956

A NEW METHOD FOR THE PREPARATION OF BOROHYDRIDES

Sir:

The authors have succeeded in preparing a series of borohydrides by the general reaction of hydrolysis of magnesium diboride, MgB_2, with bases. The MgB_2 used in this study was prepared by direct combination of boron and magnesium at 950° in a closed system under an argon atmosphere. The MgB_2 (84%) dissolved in acid solutions, leaving only small amounts of acid insoluble borides (*e.g.*, MgB_4) as a residue; it reacted exothermically with water to give hydrogen, traces of boranes, a water soluble fraction and grey water insoluble solid. The latter consisted mostly of $Mg(OH)_2$ and magnesium borates; the dark brown water soluble fraction gave off large amounts of hydrogen when acidified.

Hydrolysis of MgB_2 in strong basic media gave similar results but in KOH and $(CH_3)_4NOH$ solutions, KBH_4 and $(CH_3)_4NBH_4$, respectively, were isolated from solution. For example, 23 grams of MgB_2, (82.4%) were digested for 8 to 12 hours in 250 ml. of 3*M* KOH. The reaction mixture was kept well stirred and cooled during the addition of the MgB_2 to the base and during the first few hours of the reaction. Thereafter the reaction ran smoothly at room temperature. The water soluble fraction was rapidly filtered and slowly evaporated under vacuum. Due to their relatively low solubility the first crystals were easily separated from the remainder of the solution by filtration. Analytical data showed that this product of the hydrolysis of MgB_2 and strong KOH was KBH_4 (B: Calcd. 20.06%; Found, 19.99%). Four moles of gas per mole of KBH_4 were evolved upon acidification, in agreement with the equation $KBH_4 + H^+ + 3H_2O \rightarrow H_3BO_3 + K^+ + 4H_2$. Our observed value of $a_0^{25°} = 6.7274 \pm 0.0003$ Å. is in complete agreement with the reported value for KBH_4 of $a_0^{25} = 6.7274$ Å.[1] A 13% conversion of boron to borohydride was obtained, as determined by the amount of hydrogen evolved upon acidification of the solution. Other crystals which formed in the solution were found by analysis to be a potassium borate of the formula $KBO_2 \cdot 1\ ^1/_4\ H_2O$. The powder diffraction pattern shows principal lines having "d" values of 5.5m, 3.78m, 2.97s, 2.73m, 2.48m, 2.25s, 1.85m and 1.60m.

The hydrolysis of MgB_2 was carried out with other bases with comparable results—*e.g.*, 7.7 g. of MgB_2 reacted in 85 ml. of 4M $(CH_3)_4NOH$, was filtered, and the filtrate evaporated slowly in vacuum. The first crystalline product to separate from the solution was $(CH_3)_4NBH_4$. A powder diffraction pattern of the crystals showed a tetragonal lattice with $a_0 = 7.29$, $c_0 = 5.696$ and $c/a = 0.719$.

The experimental results show that one can produce any borohydride from the general reaction of hydrolysis of MgB_2 in a strong basic medium. The borohydride can be isolated from solution if it is stable in the basic medium at room temperature and less soluble than its borate, also present in solution. In any event one has a simple means available for the preparation of laboratory quantities of a basic solution of most borohydrides.

(1) S. C. Abrahams and J. Kalnais, *J. Chem. Phys.*, **22**, 434 (1954).

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RECEIVED JUNE 19, 1956

THE ENZYMATIC SYNTHESIS OF N[10]-FORMYLTETRAHYDROFOLIC ACID AND ITS ROLE IN ATP FORMATION DURING FORMIMINOGLYCINE DEGRADATION

Sir:

Extracts of *Clostridium acidi-urici* and *Clostridium cylindrosporum* degrade purines to formiminoglycine (NH=CH–NH–CH_2–COOH)[1] in a series of hydrolytic reactions.[2] The further metabolism of FIG by a purified enzyme preparation requires ADP, P_i and a cofactor present in boiled extracts, and leads to the formation of ATP as shown by reaction (1).[3]

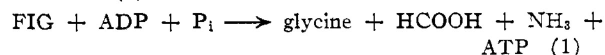


FIG + ADP + P_i ⟶ glycine + HCOOH + NH_3 + ATP (1)

The activity of the boiled extract is completely replaced by 10-formyl-THF or THF, and evidence has now been adduced for the following steps in the over-all reaction (1)

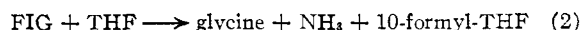
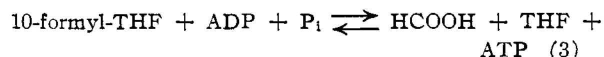


FIG + THF ⟶ glycine + NH_3 + 10-formyl-THF (2)


10-formyl-THF + ADP + P_i ⇄ HCOOH + THF + ATP (3)

With substrate amounts of 10-formyl-THF, FIG is not required for the formation of ATP (Table I) [equation (3)]. As in the over-all reaction (1), ATP cannot be demonstrated unless hexokinase, glucose and $MgCl_2$ are added as a trapping system. In the absence of the trapping system, the equilibrium lies far to the left and formic acid and THF are readily converted to 10-formyl-THF in the presence of ATP.[4]

(1) Abbreviations used are: FIG, formiminoglycine; THF, tetrahydrofolic acid; 10-formyl-THF, N[10]-formyltetrahydrofolic acid; 5-formyl-THF, N[5]-formyltetrahydrofolic acid (leucovorin or citrovorum factor); 5,10-formyll-THF, the cyclic N[5]-N[10]-imidazolinium derivative of 5-formyl-THF (anhydroleucovorin or anhydrocitrovorum factor).

(2) J. C. Rabinowitz and W. E. Pricer, Jr., *J. Biol. Chem.*, **218**, 189 (1956), and earlier references cited therein; J. C. Rabinowitz and W. E. Pricer, Jr., *ibid.*, in press.

(3) J. C. Rabinowitz and W. E. Pricer, Jr., THIS JOURNAL, **78**, 1513 (1956).

(4) The formation of 10-formyl-THF from HCOOH, ATP and THF with a purified pigeon liver extract has been described by Greenberg, *et al.*[7]