TABLE II

PYRIDOXAL PHOSPHATE REQUIREMENT FOR $H_2C^{14}O$ AND SERINE-3-$C^{14}$ INCORPORATION INTO METHIONINE

The enzymes in the samples were preincubated under $N_2$ at 37° for 75 min. with 25 μmoles ATP, 2 μmoles PLP, 12 μmoles DPX in various combinations as recorded in the table. After preincubation, additions identical with Table I were made with either serine-3-$C^{14}$ or $H_2C^{14}O$ as one-carbon sources, and incubation carried out under nitrogen at 37° for 75 min. Enzyme prepared as in Table I, stored at 4° for 20 hours.

| Additions | Counts in methionine methyl groups: Serine-3-$C^{14}$ ct./min. | $H_2C^{14}O$ ct./min. |
|---|---|---|
| ATP | 530 | 795 |
| ATP + PLP | 630 | 910 |
| ATP + DPX | 70 | 355 |
| ATP + DPX + PLP | 265 | 695 |

Berg[4] was the first to demonstrate the *in vitro* incorporation of $HC^{14}OOH$ into methionine methyl, and he concluded that the incorporation represented *de novo* synthesis. However, some questions have been raised by Sloane, *et al.*,[5] as to whether or not this represents an exchange process rather than a true synthesis. These workers, using Berg's pigeon liver extract system, were unable to demonstrate a net increase in methionine on incubating formate and homocysteine with various cofactors. In our thoroughly dialyzed system, we found (Table I) that the addition of methionine to our media greatly increased the yield of labeled methionine. This increase could result from the added methionine serving either to trap biosynthesized methionine or as a source of methionine for the exchange of its methyl carbon with the labeled one-carbon source in some manner. In Table III we include data which indicate that the incorporation of label

TABLE III

INCORPORATION OF $H_2C^{14}O$ AND SERINE-3-$C^{14}$: EXCHANGE OR SYNTHETIC PROCESS ?

Each vessel contained 20 μmoles DL-homocysteine, 1 μmole DL-methionine or DL-methionine-$C^{14}H_3$ (16,600 ct./min./μmole), 10 μmoles $MgSO_4$, 20 μmoles ATP, 2 μmoles DPN, 200 μmoles Tris at *p*H 7.3, and 1 ml. enzyme prepared as in Table I. Samples 3–5 included either 20 μmoles DL-serine or DL-serine-3-$C^{14}$ (14,100 ct./min./μmole). Samples 6–8 included either 11.4 μmoles $H_2CO$ or $H_2C^{14}O$ (16,800 ct./min./μmole) and 200 γ leucovorin. Final vol. 3 ml., incubated under nitrogen at 37° for 2 hr.

| Sample no. | Additions | Counts in methionine methyl groups ct./min. |
|---|---|---|
| 1 | DL-Methionine-$C^{14}H_3$[a] | 16,600 |
| 2 | DL-Methionine-$C^{14}H_3$ | 14,180 |
| 3 | DL-Methionine-$C^{14}H_3$, DL-serine | 14,200 |
| 4 | DL-Methionine-$C^{14}H_3$, DL-serine-3-$C^{14}$ | 19,100 |
| 5 | DL-Methionine, DL-serine-3-$C^{14}$ | 6,350 |
| 6 | DL-Methionine-$C^{14}H_3$, $H_2CO$ | 14,500 |
| 7 | DL-Methionine-$C^{14}H_3$, $H_2C^{14}O$ | 21,100 |
| 8 | DL-Methionine, $H_2C^{14}O$ | 5,780 |

[a] Zero time control.

from both $H_2C^{14}O$ and serine-3-$C^{14}$ is indeed a synthetic process. Samples 3 and 6 show that unlabeled $H_2CO$ and serine do not dilute methionine-$C^{14}H_3$ added to the reaction medium. Dilution would be expected if the one-carbon sources were exchanging with the methionine. Furthermore, the sum of labeled methionine methyl groups in samples 3 and 5, where either labeled methionine or labeled serine were used, approaches that found in sample 4 where both methionine and serine were labeled. Similarly, the sum in samples 6 and 8 approach the value found in 7. With the specific radioactivities of methionine methyl, the carbon-3 of serine and the formaldehyde–carbon being of like magnitudes, an exchange process would result in a labeled methionine recovery in samples 4 and 7 approximately equal to that in Sample 2.

The degree of incorporation of label from either serine-3-$C^{14}$ or from $H_2C^{14}O$ varied widely with different enzyme preparations and consequently the incorporations in counts per minute between experiments cannot be directly compared.

Other preliminary findings indicate that tetrahydrofolic acid (THFA) can be substituted for leucovorin when $H_2CO$ is used as substrate, but does not eliminate the ATP requirement. With serine as the one-carbon source, however, THFA has a strong inhibitory effect in our crude system. Also, at this stage, the use of reduced DPN in place of DPN does not significantly increase the yield of labeled methionine. Quite conceivably the mechanisms involved when serine serves as the one-carbon source may take two pathways. Pathway I would first entail a breakdown to a one-carbon intermediate and glycine with the subsequent addition of this one-carbon intermediate to homocysteine. This pathway would be mediated by a folic acid derivative. Pathway II would result from a condensation of serine or serine derivative with homocysteine or homocysteine derivative to form a cystathione-like compound. Cleavage of this compound would result in the formation of methionine. These possible mechanisms are being investigated.

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## A NEW CHEMICAL SYNTHESIS OF FLAVIN-ADENINE-DINUCLEOTIDE AND ANALOGS[1,2]

*Sir:*

Although several methods have been reported for the isolation of FAD[3] from natural sources, the pure compound has remained somewhat inaccessible owing to the low yields and lability of the material during isolation. Recently, the chemical synthesis of FAD has been achieved through the condensation of the mono-silver salt of FMN with 2′,3′-isopropylidene adenosine-5′-benzyl phosphochloridate.[4] This route, however, requires the removal

(1) Paper III in the series "Flavin Nucleotides and Flavoproteins"; for papers I and II see THIS JOURNAL, **74**, 5440 (1952), and **75**, 3611 (1953).

(2) Supported by research grants from Eli Lilly and Co. and Initiative 171, State of Washington.

(3) The following abbreviations will be used: FAD, flavin-adenine-dinucleotide; FID, flavin-inosine-dinucleotide; FMN, riboflavin-5′-phosphate; *cyc*-FMN, riboflavin-4′,5′-(cyclic)-phosphate; RbPP, riboflavin-5′-pyrophosphate; AMP, adenosine-5′-phosphate; IMP, inosine-5′-phosphate; DPTC, di-*p*-tolyl carbodiimide.

(4) S. M. R. Christie, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 46 (1954).

of protective groups after the labile pyrophosphate bridge has been formed, which reduces the over-all yield to about seven per cent.

During an investigation of the chemical synthesis of FAD through a route involving no protective groups,[5] RbPP was prepared by several methods, *inter alia* by the reaction of FMN with $H_3PO_4$ in the presence of a carbodiimide. The effectiveness of this method for preparing RbPP as well as the previous use of carbodiimides in the formation of pyrophosphate esters,[6,7,8] suggested the possibility of synthesizing the pyrophosphate link of FAD *directly* by removal of the elements of water from FMN and AMP.

A typical preparation, based upon optimal conditions, may be summarized as follows: 200 mg. of FMN (sodium salt) and 800 mg. of AMP (free acid) are dissolved in 2 ml. of water and 10 ml. of pyridine, and to this mixture is added a solution of 8 g. of DPTC in 10 ml. of pyridine. The resulting mixture is shaken in the dark for 24 hours, 100 ml. of water added, and the insoluble carbodiimide and di-*p*-tolyl urea are removed by filtration. The flavin mixture is concentrated by lyophilization and chromatographed (descending) on 50 × 70 cm. sheets (*ca.* 100 mg. material per sheet) of Munktell, Cremer-Tiselius paper[9] with *n*-butanol:acetic acid:water (4:1:5, organic phase) as the developing solvent. The slowest moving component (FAD, $R_f$ = 0.12) is eluted with water and lyophilized to yield 14 mg. of FAD ($P$ = 0.06, $R$ = 48).[10] This material is purified further on Munktell paper (descending) using *t*-butanol: water (60:40) as the solvent (FAD, $R_f$ = 0.40). After the material has been chromatographed twice in the *t*-butanol:water system, 4.7 mg. of FAD ($P$ = 0.40, $R$ = 3.5) are obtained. The material is now free from adenosine compounds and is over 90% pure on a flavin basis as ascertained in the D-amino acid oxidase system.[11,12] Final purification of synthetic FAD *via* the uranyl salt follows the method for the natural product as described elsewhere.[12]

The pure synthetic FAD shows only a single, homogeneous spot when subjected to paper chromatography in a variety of solvent systems.[11] From the absorption spectrum of the material at *p*H = 7.0, an $R$ value of 3.3 is observed.[13]

The appearance of *cyc*-FMN as the principal reaction product was not unexpected in view of the ease with which FMN undergoes cyclization in the presence of dehydrating agents. Carbodiimides have been shown to promote cyclization in other nucleotides.[14,15] When the more reactive dicyclohexyl carbodiimide[6] replaced the di-*p*-tolyl derivative, *cyc*-FMN was obtained as the sole product.

This method affords a convenient, one-step synthesis of modest amounts of pure FAD from commercially available materials. It provides also a convenient route to the synthesis of otherwise unobtainable analogs of FAD wherein the AMP portion is replaced by other purine or pyrimidine nucleotides. Thus, FID has been prepared by the condensation of FMN and IMP. The purified material moves as a single spot ($R_f$ = 0.72) upon paper chromatography in 0.1 *M* acetate buffer, *p*H = 4.0 (FAD, 0.61; FMN, 0.57), and is identical with the product obtained by deamination of FAD with nitrous acid. FID is devoid of coenzymatic activity in the D-amino acid oxidase system.

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(8) E. P. Kennedy and S. B. Weiss, THIS JOURNAL, **77**, 250 (1955).

(9) Supplied by E. H. Sargent and Co., 4647 West Foster Ave., Chicago, Ill.

(10) $P$ = dry weight purity determined on a sample dried at 65° *in vacuo* over $P_2O_5$ and assuming $\epsilon = 11.3 \times 10^3$ sq. cm./mole at 450 mμ. Molecular weight of FAD (free acid) is 785.6. $R$ = ratio of light absorption 260: 450 mμ at *p*H 7.0.

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