TABLE I

PAPER CHROMATOGRAPHY OF THYMIDYLIC ACID OBTAINED BY ACID HYDROLYSIS OF THE THYMIDINETRIPHOSPHATE PEAK[a]

System	Rf Sample	Rf TMP
Isopropyl alcohol–HCl[b]	0.91	0.91
Isobutyric acid–ammonia[c]	.59	.59
N-Propyl alcohol–ammonia[d]	.26	.29

[a] Hydrolysis, 10 minutes in *N* HCl at 100°. [b] G. R. Wyatt, *Biochem. J.*, **48**, 584 (1951). [c] See reference (9). [d] C. S. Hanes and F. A. Isherwood, *Nature*, 164, 1107 (1949).

TDP which were separated by ion-exchange chromatography.

TABLE II

PAPER CHROMATOGRAPHY OF A BASE FROM THE THYMIDINETRIPHOSPHATE PEAK[a]

System	Rf Sample	Rf Thymine
Isopropyl alcohol–HCl[b]	0.79	0.79
Butyl alcohol–water[c]	.52	.52
Isobutyric acid–ammonia[d]	.81	.81

[a] An aliquot of the TMP sample obtained by acid hydrolysis of the TTP peak (*cf.* Table I) was hydrolyzed 1 hour in 98% formic acid at 175° in a sealed tube. [b] See footnote (*b*) Table I. [c] R. Markham and J. D. Smith, *Biochem. J.*, **45**, 294 (1949). [d] See reference (9).

TTP obtained from another chromatogram was purified by paper chromatography.[9] The isolated material with a D_{275}/D_{260} ratio of 0.92 contained per μM. of TTP: deoxyribose, 0.75 μM.; acid-labile P, 1.55 μM.; and total P, 2.47 μM. Deoxyribose: acid-labile P: total P = 0.92:1.88:3.00.

The only evidence for TDP *per se* was the appearance of 2.0 μM. of TMP in an enzymatic (apyrase) hydrolysate of the ATP peak. Chromatographic evidence[11] and technical considerations[6,12] suggest that TDP, if it exists, would appear in or near the ATP peak.

Thymidylic acid (2.0 μM.) was found by rechromatography of the UMP peak (AM-F system): deoxyribose, 1.02 μM./μM. of TMP and total P, 0.97 μM./μM. of TMP.

Acid hydrolysis of the CTP peak gave a mixture of cytidylic acids which could be resolved into CMP and D-CMP by either ion-exchange using the borate technique[13] or paper chromatography.[10] In addition, paper chromatography[9] of the CTP peak purified on the AM-F system[6] gave only two bands, each of which had identical spectra in acid (maximum at 280 mμ, minimum at 242 mμ) and alkali. The faster moving band contained per μM. of D-CTP: deoxyribose, 0.86 μM.; acid-labile P, 1.75 μM.; and total P, 2.62 μM.

Rechromatography of the CDP peak in the AM–F system gave what appeared to be only CDP 10.4 μM.). A Brody test, however, indicated 0.41 μM. of deoxyribose/μM. of nucleotide. Paper chromatography[9] resolved the mixture into CDP and D-CDP. The latter contained: deoxyribose, 0.87 μM.; acid-labile P, 0.87 μM.; and total P, 1.8 μM. (data expressed per μM. of D-CDP).

By the same technique the CMP peak yielded about one μM. of D-CMP.

It should be pointed out that all of the cytidine nucleotide preparations so far examined contained deoxyribose and ribose in roughly a 40:60 ratio.

No evidence for deoxypurine nucleotides has been found.

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(10) H. A. Krebs and R. Hems, *Biochim. et Biophys. Acta*, **12**, 172 (1953).

(11) Data to be presented in a more complete publication.

(12) L. I. Hecht, V. R. Potter and E. Herbert, *Biochim. et Biophys. Acta*, **15**, 134 (1954).

(13) J. X. Khym and W. E. Cohn, *ibid.*, **15**, 139 (1954).

CO-FACTOR REQUIREMENTS FOR THE INCORPORATION OF $H_2C^{14}O$ AND SERINE-3-C^{14} INTO METHIONINE[1]

Sir:

Using $H_2C^{14}O$ and serine-3-C^{14} as one-carbon sources, we have investigated the co-factor requirements for the *in vitro* incorporation of these compounds into methionine methyl groups by cell-free extracts of sheep liver. The methyl groups of methionine were assayed by cleavage with HI[2] and trapping the evolved CH_3I with trimethylamine.[3] The requirements for Mg^{++}, ATP, DPN and homocysteine have been shown with either H_2CO or serine as the one-carbon source, whereas, the co-factor effect of leucovorin is apparent only when H_2CO is utilized (Table I). The requirement for pyridoxal phosphate (PLP), although not apparent from the data of Table I, is demonstrated in Table II where data for the pre-incubation of enzyme with PLP, ATP, and deoxypyridoxine (DPX) in various combinations indicate that the inhibition of incorporation of both $H_2C^{14}O$ and serine-3-C^{14} by DPX can be partially reversed by PLP.

TABLE I

CO-FACTOR REQUIREMENTS FOR THE INCORPORATION OF SERINE-3-C^{14} AND $H_2C^{14}O$ INTO METHIONINE

Each vessel contained 20 μmoles DL-homocysteine, 20 μmoles DL-serine-3-C^{14} (14,100 ct./min./μmole) or 11.4 μmoles $H_2C^{14}O$ (16,800 ct./min./μmole), 1 μmole L-methionine, 10 μmoles $MgSO_4$, 20 μmoles ATP, 2 μmoles DPN, 1 μmole pyridoxal phosphate, 400 γ leucovorin, 200 μmoles tris-(hydroxymethyl) aminomethane (Tris) at *p*H 7.3, and 1 ml. enzyme. Enzyme was prepared by homogenizing 1 part sheep liver with 2 parts Tris buffer, 0.1 *M*, *p*H 7.3, and dialyzing the supernatant fluid obtained by centrifuging at 100,000 × *g* against 0.025*M* Tris buffer for 24 hours. Final vol. 3 ml., incubated under nitrogen at 37° for 2 hours.

Omission	Counts in methionine methyl groups: Serine-3-C^{14} ct./min.	$H_2C^{14}O$ ct./min.
None	15,650	3800
Homocysteine	220	380
Methionine	3,660	780
$MgSO_4$	320	85
ATP	55	0
DPN	4,430	925
Pyridoxal phosphate	14,400	4210
Leucovorin	13,900	1070

(1) Aided by research grants from the National Institutes of Health, U. S. Public Health Service, and the California Division of the American Cancer Society.

(2) H. D. Baernstein, *J. Biol. Chem.*, **106**, 451 (1934); **115**, 25 (1936).

(3) W. Sakami, *ibid.*, **187**, 369 (1950).

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).

(4) P. Berg, *J. Biol. Chem.*, **205**, 145 (1953).

(5) N. H. Sloane, E. Boggiano, B. Smith, B. L. Hutchings, *Fed. Proc.*, **14**, 282 (1955).