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Tetrahydrofolate Cofactors in Tissues Sensitive and Refractory to Amethopterin*

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ABSTRACT: Tetrahydrofolate cofactors in extracts of two tumors, liver, and intestinal mucosa were separated by column chromatography using DEAE-cellulose and were measured by microbial assays. These tissues were studied because of differences in their sensitivity to treatment with amethopterin and to dietary deficiency of folic acid. The distribution pattern of the tetrahydrofolate cofactors differed in each tissue. The ratio of 10-formyltetrahydrofolate:5-methyltetrahydrofolate was reversed in the two tumors (7:1 in the Murphy Sturm lymphosarcoma and 1:4 in the Walker carcinoma 256). Also, the Murphy Sturm lymphosarcoma, which is sensitive to amethopterin, was characterized by a higher content of total free folic acid activity than the Walker carcinoma 256 which is quite refractory to amethopterin. The major derivative found in liver was 5-methyltetrahydrofolate whereas the elution profile of intestinal mucosa showed almost equivalent amounts of 10-formyltetrahydrofolate and 5-methyltetrahydrofolate.

Derivatives of tetrahydrofolate function as cofactors essential for the biosynthesis of nucleic acids (Friedkin, 1963), yet little is known about their relative concentrations in normal and neoplastic tissues. Such compounds have been identified and assayed in several mammalian tissues (Donaldson and Keresztesy, 1959; Usdin, 1959; Herbert *et al.*, 1962; Silverman *et al.*, 1961; Noronha and Silverman, 1962) as well as in plants (Iwai and Nakagawa, 1959). Variation in the dependence of different tissues on the availability of folic acid cofactors is indicated by the leucopenia and desquamation of mucosal membranes in animals depleted of this vitamin (Delmonte and Jukes, 1962). Also, some selective impairment of different cells is recognized by the remission of acute lymphocytic leukemia in patients treated with amethopterin (Methotrexate) and by the erosion of mucosal surfaces in some subjects treated with this drug (Holland, 1961).

A difference in nutritional requirement for the growth of two tissues was shown by the failure of Walker carcinoma 256 to grow in rats only moderately depleted of folic acid (Rosen and Nichol, 1962), in contrast to the normal growth rate of Murphy Sturm lymphosarcoma even in severely depleted animals (Rosen *et al.*, 1964). It was of interest, therefore, to determine whether there was any difference between these experimental tumors with regard to their content of tetrahydrofolate derivatives. Also, the sensitivity of intestinal mucosa to amethopterin can be contrasted to the lack of any hepatotoxicity of this drug. Consequently, these four tissues were selected for the comparative study described herein. A preliminary report of this work has been presented (Sotobayashi *et al.*, 1965).

Materials and Methods

Reference Compounds. Folic acid and calcium leucovorin were purchased from the American Cyanamid Co., and tetrahydrofolic acid (H_4-folate) from General Biochemicals, Inc. 10-Formyl-H_4-folate was prepared by isomerization of 5-formyl-H_4-folate with dilute HCl at 0° and neutralization to pH 6.0 (May *et al.*, 1951; Cosulich *et al.*, 1952). 5-Methyl-H_4-folate was

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prepared by the reduction of methylene-H_4-folate (Sakami, 1963). 5,10-Methylene-H_4-folate was prepared by the procedure of Osborn *et al.* (1960).

Extraction of Tissues. The experimental tumors (Murphy Sturm lymphosarcoma and Walker carcinoma 256) have been maintained in this laboratory for several years by serial transplantation and are the same lines used previously (Rosen *et al.*, 1964). Male rats weighing from 100 to 125 g (Holtzman Rat Co., Madison, Wis.) were fed a stock diet (Teklad) or purified diets prepared as previously described (Rosen and Nichol, 1962). Solid pieces (1.5 mm^3) were cut from 10- to 14-day-old tumors and implanted by trocar into the flank of rats. After 8–10 days, the rats were killed and the tumors were dissected free from any subcutaneous or necrotic tissues. Intact livers or whole lobes were taken from rats after rapid exsanguination. Intestinal mucosa was scraped from thoroughly washed and blotted segments of the small intestine. Pooled samples of the tissues from four to six rats were used in most experiments. All tissues were chilled promptly and were kept cold until extracted.

The tissues were homogenized in a Waring blendor using ice-cold 1% potassium ascorbate, pH 6.0, as the diluent to prevent oxidation of the H_4-folate cofactors. To extract the tetrahydrofolate derivatives, the homogenates (1:10) were then heated for 30 min at 75° since very little isomerization of 10-formyl- to 5-formyl-H_4-folate occurs at this temperature (Silverman *et al.*, 1961). The solutions were cooled to 0° and centrifuged. The clear supernatant fluid was carefully decanted and used directly for the chromatographic separations and microbiological assays. The samples were held in an ice bath immediately after preparation or frozen if held overnight.

Chromatographic Fractionation of Tetrahydrofolate Derivatives. This procedure is a modification of that used by Silverman *et al.* (1961). A mixture of 9 g of *N,N*-DEAE-cellulose (Eastman Organic Chemicals) and 11.3 g of purified Celite No. 545 (Johns-Manville Corp. (Hall, 1962) was suspended in 750 ml of water and packed into a glass column by gravity flow. The columns were washed first with 25 ml of 0.5 N KOH followed by water until the rinse was neutral, and then with 25 ml of 0.5 M potassium phosphate buffer, pH 6.0, followed by water until the rinse was free of phosphate. The columns used were 1.5 cm in width and about 20 cm in height. Each tissue extract (1–5 ml) was placed on the column and this was washed into the column by two 2-ml portions of 0.6% of potassium ascorbate, pH 6.0. This ascorbate solution (10 ml) was then placed on the column and 0.5 M potassium phosphate buffer, pH 6.0, containing 0.6% of ascorbate was passed dropwise through a mixing chamber which contained 200 ml of 0.6% of potassium ascorbate, pH 6.0. Fractions (5 ml) were collected in tubes containing 30 mg of potassium ascorbate (pH 6.0). The flow rate was about 1 ml/min. The chromatography was performed at room temperature and the fractions were kept frozen until the samples were to be diluted for microbiological activity.

Microbiological Assay. Both *Lactobacillus casei* (ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081) were used as test organisms for assaying tissue extracts and the effluents from columns for folic acid derivatives. The medium described by Herbert (1961) was used for *L. casei* and that for *P. cerevisiae* was purchased from the Difco Laboratories. The fractions and extracts were diluted, if necessary, with 1% potassium ascorbate, pH 6.0. Aliquots of 0.02–0.1 ml were added to each assay tube. Potassium ascorbate was added so that the medium contained 2–3 mg/ml. There was a slight growth stimulation that was uniform within this range of concentration of ascorbate. The assay tubes were steamed at 100° for 20 min, cooled, and inoculated with the organism. The tubes were incubated at 37° for 16–19 hr; the turbidity was measured with a Klett–Summerson photoelectric colorimeter using a red filter. Calcium leucovorin (5-formyl-H_4-folate) was used as a reference standard for both organisms. The concentration of this standard was adjusted to correct for the presence of the inactive isomer. Chicken pancreas conjugase (Difco Laboratories) was used to detect any polyglutamate derivative (Kazenko and Laskowski, 1948).

Results

The sequence in which the folic acid derivatives were eluted from the DEAE-cellulose column was verified by the use of the reference compounds. 10-Formyltetrahydrofolate was clearly separated as the first peak. Although 5-methyl-H_4-folate was sometimes partially separated from 5-formyl-H_4-folate, in most cases these two compounds were eluted together. Fortunately, the inadequate separation by chromatography was compensated for by the different activity for the two microorganisms. 5-Formyl-H_4-folate has the same activity for *L. casei* and *P. cerevisiae* but the activity of 5-methyl-H_4-folate for *P. cerevisiae* is only 2.5% of that for *L. casei*. Consequently, the distribution pattern was based on the activity in each collecting tube measured by both organisms and the amount of 5-methyl-H_4-folate was determined by the differential

TABLE I: Elution Sequence of Folic Acid Derivatives from DEAE-Cellulose Column.

Compound	Tube No. Containing Eluted Compd	Vol. of Effluent (ml)
10-Formyl-H_4-folate	14–18	70–90
5-Methyl-H_4-folate	21–26	105–130
5-Formyl-H_4-folate	24–28	120–140
Tetrahydrofolate	28–32	140–160
5,10-Methylene-H_4-folate	37–41	185–205
Folic acid	>48	>240

TABLE II: Total Folic Acid Content and Amounts of Tetrahydrofolate Cofactors in Normal and Neoplastic Tissues of Rats.[a]

Tissue	Total Free Folic Acid Act. (mμg/g wet wt)	Tetrahydrofolate Cofactors (mμg/g wet wt of tissue)				
		10-Formyl	5-Methyl	5-Formyl	5,10-Methylene	H_4-Folate
Walker carcinoma 256	620	56 (9.0)[b]	198 (32.0)	30 (4.8)	—	183 (30.0)
	890	49 (5.5)	202 (22.7)	16 (1.8)	—	215 (24.2)
	973	85 (8.7)	229 (23.5)	30 (3.1)	—	249 (25.6)
Murphy Sturm lymphosarcoma	2,538	1,312 (51.6)	156 (6.1)	432 (17.0)	—	208 (8.2)
	4,690[c]	1,728 (36.8)	323 (6.9)	982 (21.0)	—	720 (15.3)
Intestinal mucosa	2,130	574 (26.9)	459 (21.5)	129 (6.1)	—	322 (15.1)
Liver	25,200	6,826 (27.1)	14,993 (59.5)	1,134 (4.5)	1,613 (6.4%)	1,352 (5.4)
	8,960[d]	1,595 (17.8)	4,649 (52.0)	349 (3.7)	561 (6.3%)	1,086 (12.1)

[a] The methodology for extraction, microbial assays, and gradient chromatography on DEAE-cellulose columns is described in the text. [b] Values in parentheses represent per cent of total activity. [c] A synthetic diet containing 6.25 mg of folic acid/kg of diet was fed. [d] A synthetic diet containing 1.25 mg of folic acid/kg of diet was fed.

microbial assay of the eluates. In most cases, H_4-folate was satisfactorily separated but sometimes overlapped the trailing edge of the preceding fraction. 5,10-Methylene-H_4-folate and folic acid were held back on the column and were well separated from each other and from the previously eluted compounds. For convenience of comparison with the distribution patterns for the different tissues, the elution sequence of the reference compounds is shown in Table I.

The recovery of known samples of synthetic H_4-folate after chromatography was 80–90%. Chromatography of a freshly opened vial of a commercial preparation of H_4-folate showed the presence of 10-formyl-H_4-folate (8.8%), 5-formyl-H_4-folate (2.5%), and folic acid (8.6%). Since the amounts of these compounds remained unchanged after treatment of another portion of the same sample at 75° for 30 min, it is unlikely that isomerization of the 10-formyl to 5-formyl derivative occurred during the extraction procedure. Such isomerization was found to be negligible by Silverman *et al.* (1961) under these conditions. However, it is to be expected that the 10-formyl-H_4-folate peak includes not only the amount of this compound originally present in the tissues but also that derived from any 5,10-methenyl-H_4-folate and 5-formimino-H_4-folate during the extraction procedure. The treatment of the different fractions with chicken pancreas conjugase (Kazenko and Laskowski, 1948) did not increase activity or result in the appearance of new compounds with folic acid activity.

The total free folic acid content and the amounts of tetrahydrofolate derivatives in liver, intestinal mucosa, and the two tumors are listed in Table II. The extracts were prepared from pooled samples of the tissues from rats which were fed a commercial pelleted diet unless otherwise indicated. In addition to the folic acid activity measured by *L. casei*, a portion of each extract was subjected to gradient chromatography and subsequent microbial assay of each fraction. Comparison of the assays in three separate experiments using Walker carcinoma 256 indicates that reproducibility of the procedure is sufficiently consistent to establish a definite pattern for the distribution of the H_4-folate cofactors (Table II). Analysis of the Murphy Sturm lymphosarcoma (MSL) from rats which were fed a synthetic diet containing an ample amount of folic acid (6.25 mg/kg) indicated a higher content of the vitamin but the distribution pattern (the predominant fraction in the form of 10-formyl-H_4-folate and minor amounts of 5-methyl-H_4-folate) was similar. Also, analysis of livers from rats which were fed a synthetic diet containing an amount of folic acid (1.25 mg/kg) resulting in partial deficiency of the vitamin indicated the presence of a reduced amount of total folic acid activity but the distribution pattern showing the predominance of 5-methyl-H_4-folate was quite similar to that of liver from animals fed the stock diet.

Differences in the distribution of tetrahydrofolate cofactors in the extracts of different tissues (pooled samples from 4 to 6 rats each) can be clearly identified by drawing an elution profile based on the microbial assays of each 5-ml fraction eluted from the DEAE-cellulose column. A main objective of this investigation was to establish whether or not the H_4-folate elution profile could provide a "fingerprint" at all characteristic of a particular tissue. By examination of the relative, rather than the absolute, amounts of H_4-folate cofactors, several features are noteworthy in the data presented in Figure 1. The H_4-folate profile of the

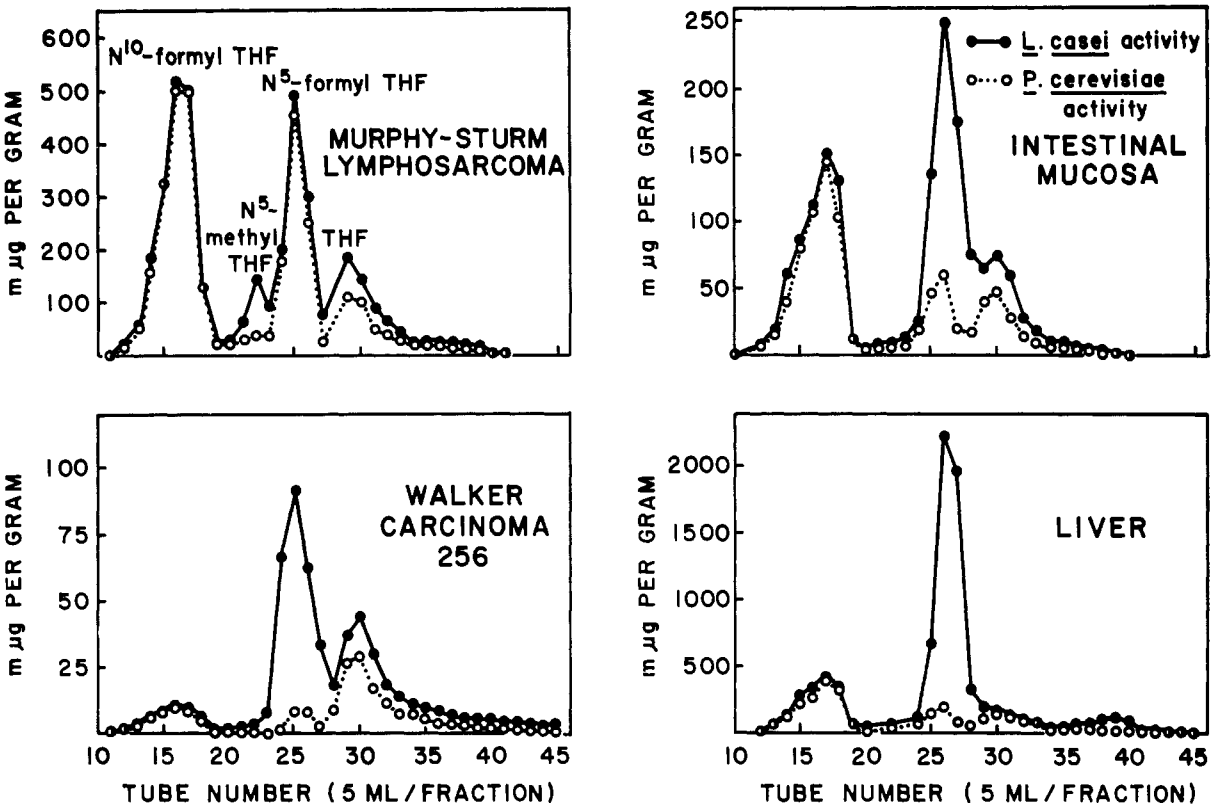

FIGURE 1. Elution patterns of tetrahydrofolate (THF) cofactors in different tissues.

ymphosarcoma differs markedly from that of the carcinoma with regard to (1) the ratio of 5-methyl to the 10-formyl cofactor for each tumor, (2) the predominance of 10-formyl-H_4-folate in Murphy Sturm lymphosarcoma, and (3) the predominance of 5-methyl-H_4-folate in the Walker tumor. The elution profile of liver is characterized by the very large fraction of 5-methyl-H_4-folate whereas the profile of intestinal mucosa shows almost equivalent amounts of the 10-formyl and the 5-methyl cofactors.

Discussion

Several factors which may condition the amount and type of tetrahydrofolate cofactors in cells require separate consideration: (i) the level of N^5-methyl-H_4-folate and other forms of folic acid in the blood and body fluids; (ii) the rate of cellular uptake of folic acid and its derivatives; (iii) the level of enzymes which mediate the formation of tetrahydrofolate and the interconversion of the cofactors; and (iv) the actual requirement for the individual cofactors in biosynthetic reactions related to the growth rate of the tissue. That the relative amounts of the tetrahydrofolate cofactors show a consistent pattern is indicated by the data in Table II for three separate experiments on the Walker tumor.

The differences in the elution profiles of Walker 256 and Murphy Sturm lymphosarcoma (Figure 1) do not appear to be related to the content of folate reductase since Werkheiser (1962, 1963) found that the level of this enzyme was similar in these two tumors. Silverman and co-workers (1961) fractionated the folate derivatives in extracts of acetone powder preparations of two experimental leukemias (a mast cell neoplasm P-815 and a plasma cell line 70429). In each case, 10-formyl-H_4-folate accounted for most of the vitamin and other folate cofactors were found in relatively smaller amounts. It is not known whether this pattern is related to a higher level of the formate activating enzyme but increased activity of this enzyme occurs in human leukemic cells (Bertino *et al.*, 1963). The ineffectiveness of amethopterin against the Walker tumor and its sensitivity to dietary deficiency of folic acid appears to be related to impaired capacity for the cellular uptake of both tritiated amethopterin and tritiated folic acid (Nichol *et al.*, 1966).

The gradient elution technique described in this report gave better resolution of the tetrahydrofolate cofactors than was obtained previously (Silverman *et al.*, 1961) using similar DEAE-cellulose column chromatography. In agreement with the report by Silverman *et al.* (1961), the detectable folic acid derivatives in the tissue extracts examined were found to be in the monoglutamate form. This conclusion is based on the observation that treatment of the different fractions with chicken pancreas conjugase preparations did not increase activity or result in the appearance

of new compounds with folic acid activity. That the enzyme preparation was active, was indicated by an increase in folic acid activity when heat-inactivated liver homogenates were treated similarly. No attempt was made to analyze the tissues for folate polyglutamates since interconversion of the tetrahydrofolate derivatives cannot be prevented during the incubation of tissue homogenates with conjugase enzyme preparations. The use of ascorbate as a protective agent in the extraction and elution solvents is preferable to 2-mercaptoethanol in view of the recent report by Zakrzewski (1966) that 2-mercaptoethanol can form complexes with tetrahydrofolate even in dilute solutions. It is not known whether such complexes account for some of the multiple forms of folate eluted by solutions containing 0.2% mercaptoethanol from DEAE-cellulose columns charged with extracts of chicken liver (Noronha and Silverman, 1962).

The number of tissue extracts which can be assayed by the methods described is restricted by the time-consuming procedure, primarily because of dependence upon microbial assays requiring the use of two organisms. If better separation of the 5-methyl-H_4-folate fraction can be accomplished by modifications of the chromatographic technique, then radioactivity measurements of the tetrahydrofolate compounds present in tissues after the administration of tritiated folate would be a preferable procedure which might be better suited to the study of the rate of formation or the rate of interconversion of these tetrahydrofolate derivatives.

Previous studies from several laboratories indicated that folic acid is stored mainly in the liver and that 5-methyl-H_4-folate is the predominant form of the vitamin in this tissue. The factors influencing or regulating the interconversion of the tetrahydrofolate compounds in this organ have not yet been defined. The substantial fraction of 5-methyl-H_4-folate in intestinal mucosa and the Walker tumor requires consideration of the functional significance of this form of the vitamin and the activity of enzymes necessary for its utilization (Buchanan, 1964).

It is of particular interest that of the tissues examined, a compound presumed to be 5,10-methylene-H_4-folate on the basis of elution sequence and microbial activity was detected only in liver (Table II). Differences among tissues other than liver are likely to be more meaningful in studying the essential functions of folic acid cofactors since the liver acts as a reservoir of the vitamin, is not growing (unless after partial hepatectomy) and is not affected by doses of amethopterin which are toxic for other tissues.

Even among tissues other than liver, distinctive features of the distribution pattern of the tetrahydrofolate cofactors are noteworthy when compared on the basis of per cent of total folic acid activity (see numbers in parentheses, Table II). The Murphy Sturm lymphosarcoma was characterized by a very low proportion (6–7%) of 5-methyl-H_4-folate in contrast to the other tissues. The Walker carcinoma was characterized by a very low content of 10-formyl-H_4-folate (5–9%). The elution profile of intestinal mucosa differed from that for each of the tumors by the similar amounts of 10-formyl-H_4-folate and 5-methyl-H_4-folate (Figure 1). Much further work is necessary to establish whether such differences are characteristic of individual tissues or related to their response to amethopterin. The results presented provide a basis for studying the conditions which can influence or regulate the tissue content of tetrahydrofolate cofactors and for comparison of the level of enzymes involved in the formation and utilization of these cofactors in tissues affected by a deficiency of folic acid or sensitive to folic acid antagonists.

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Coenzyme A Analogs. II. Enzymatic Conversion of D-Oxypantetheine 4′-Phosphate to Oxy-Coenzyme A*

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ABSTRACT: The enzymatic conversion of D-oxypantetheine 4′-phosphate to oxy-coenzyme A was investigated with coenzyme A synthesizing enzymes isolated from beef liver. DEAE-cellulose column chromatography of the enzymatic incubation mixtures containing ATP-8-^{14}C and D-oxypantetheine 4′-phosphate separated a radioactive component identical with synthetic oxy-coenzyme A. Dephosphooxy-coenzyme A was enzymatically (a) cleaved by pyrophosphate to oxypantetheine 4′-phosphate and ATP and (b) phosphorylated by ATP to oxy-coenzyme A. Enzymatically prepared oxy-coenzyme A possessed the same inhibitory action as the synthetic material in the phosphotransacetylase reaction.

The oxygen analog of coenzyme A, oxy-coenzyme A, has been recently synthesized and shown to be a competitive inhibitor of CoA[1] in the phosphotransacetylase reaction (Stewart and Miller, 1965; Miller *et al.*, 1966). In order to determine if oxy-CoA can be prepared enzymatically, we investigated the ability of the crude CoA-synthesizing enzyme system, isolated from beef liver by the procedure of Hoagland and Novelli (1954), to convert D-oxypantetheine 4′-phosphate to oxy-CoA.

A net synthesis of oxy-CoA was achieved *via* reactions 1 and 2.

oxypantetheine 4′-phosphate + ATP = dephosphooxy-CoA + PP_i (1)

dephosphooxy-CoA + ATP → ADP + oxy-CoA (2)

(net) oxypantetheine 4′-phosphate + 2 ATP → oxy-CoA + ADP + PP_i

Experimental Procedures

Materials. D-Oxypantetheine 4′-phosphate, dephosphooxy-CoA, and oxy-CoA were prepared by the procedures of Miller *et al.* (1966). D-Pantetheine 4′-phosphate was prepared by the procedure of Moffatt and Khorana (1961). ATP-8-^{14}C (lot no. 6501) was purchased from Schwarz Bioresearch, Inc., and unlabeled ATP was purchased from Sigma Chemical Co. DEAE-cellulose (Selectacel standard type) was obtained from Carl Schleicher and Schuell. Phosphotransacetylase (lot no. 06155109) CoA, and acetyl phosphate were purchased from Boehringer Mannheim Corp. Lyophilized *Clostridium kluyveri* cells, as a source of crude transacetylase, and venom phosphodiesterase (*Crotalus adamantens*) were purchased from Worthington Biochemical Corp.

Methods. The Biuret reaction was used for protein determinations (Layne, 1957). Venom phosphodiesterase digestions of CoA and oxy-CoA were done by the previously described procedures (Moffatt and Khorana, 1961; Miller *et al.*, 1966). Phosphotransacetylase assays were performed by the procedure of Stadtman (1952) when preparing the beef liver enzymes, and as modified by Bergmeyer *et al.* (1963) for the inhibition studies. The ^{14}C content of column fractions was determined by adding 0.1-ml aliquots to 14 ml of the aqueous counting medium of Bray (1960) and

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[1] Abbreviations used: oxy-CoA, oxy-coenzyme A; desulfo-CoA, desulfocoenzyme A; dephospho-CoA, 3′-dephosphocoenzyme A; dephosphooxy-CoA, 3′-dephosphooxy-coenzyme A; AMP and ATP, adenosine mono- and triphosphates. The IUB systematic name for each of the following trivial enzyme names employed in this paper is: phosphotransacetylase, acetyl-CoA; orthophosphate acetyltransferase (EC 2.3.1.8); dephospho-CoA pyrophosphorylase, ATP:pantetheine 4′-phosphate adenylyltransferase (EC 2.7.7.3); dephospho-CoA kinase, ATP:dephospho-CoA 3′-phosphotransferase (EC 2.7.1.24).