STUDIES ON THE PHYSIOLOGY OF RICKETTSIAE
IV. FOLIC ACIDS OF *COXIELLA BURNETII*

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Received for publication 23 July 1962

ABSTRACT

MATTHEIS, MARTHA S. (University of Kansas, Lawrence), M. SILVERMAN, AND D. PARETSKY. Studies on the physiology of rickettsiae. IV. Folic acids of *Coxiella burnetii*. J. Bacteriol. **85**:37–41. 1963.—Yolk, yolk sac, and embryo tissues of uninfected eggs, and those infected with *Coxiella burnetii*, were analyzed for folic acid derivatives by employing diethylaminoethyl (DEAE)-cellulose column chromatography. Infected tissues contained quantitatively less folate, but the elution profiles of both infected and uninfected tissues were identical. Purified *C. burnetii* contained some types of folate apparently unique to these rickettsiae, and not found in infected tissue. The major folate fraction of *C. burnetii* was partially characterized by (i) elution position from DEAE columns; (ii) treatment with conjugase; (iii) growth response by *Lactobacillus casei*, *Streptococcus faecalis* R, and *Pediococcus cerevisiae*; and (iv) response to oxidation, reduction, and formylation.

Folic acid or its derivatives are widely distributed among multicellular and unicellular forms, in free-living as well as in parasitic microorganisms. The biochemical functions of the folic acid series have been well reviewed (Huennekens and Osborn, 1959; Rabinowitz, 1960), but there is little information on the significance of the presence and function of folates in parasitic organisms. Folic acid was demonstrated in the rickettsiae *Rickettsia mooseri* (Kleinschmidt, Holmes, and Behrens, 1956) and *Coxiella burnetii* (Myers, 1958), in the malarial parasite *Plasmodium lophurae* (Trager, 1959), and in the psittacosis group of viruses (Colón and Moulder, 1958; Colón, 1960, 1962). Dewey and Kidder (1960) showed that the ciliate *Tetrahymena geleii* utilized folic acid in transformylation, and Myers and Paretsky (1961) demonstrated transformylation in *C. burnetii* in the presence of tetrahydrofolate. Trager's (1959) observations on the enhanced folic and folinic acid content of duck red blood cells infected with *P. lophurae* suggest a possible difference between plasmodial and erythrocyte folate forms. Silverman, Law, and Kaufman (1961) showed that the folate distribution patterns of leukemic cells and of the host mouse liver differed significantly. The presence and activity of folate in *C. burnetii* suggest need for a closer study of the folic acid forms of *C. burnetii* and the host chick embryo tissues.

MATERIALS AND METHODS

C. burnetii was propagated in yolk sacs, using an inoculum of the second and third yolk-sac passage of a guinea pig-adapted Nine Mile strain. The organisms were propagated, harvested, purified, and titered as described by Paretsky et al. (1958, 1962).

Tissue homogenates of infected and uninfected chick embryonic tissue were prepared by pooling 8 to 12 samples of each tissue and homogenizing in a cold Waring Blendor for 1 to 2 min. In the 10- and 15-day-old embryos, the heads and feet were first excised.

Uninfected 10- and 15-day-old yolk sacs were purified at the same time as the infected material. Samples were removed for folate assay at various steps of purification.

Acetone powders prepared from the several homogenates and from purified rickettsiae were stored in a desiccator at −20 C. The powders were prepared and extracted at 75 C as described by Romine (1960) and Silverman et al. (1961). Acetone powder extracts were chromatographed on diethylaminoethyl (DEAE)-cellulose columns by the procedures of Silverman et al. (1961),

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TABLE 1. *Elution pattern of folic acid and related compounds from DEAE-cellulose columns*

Compound	Tube no. (peak)
Ptero-2-glutamic acid*	9
N^5,N^{10}-Isoleucovorin	9
N^{10}-Formylfolic acid	11
N^5-Formyltetrahydrofolic acid	11
Yolk-sac fraction	11, 12
Tetrahydrofolic acid	12
Adult chicken liver fractions	11, 16
Beef-liver fraction	13
Chicken-embryo fractions	11, 16
N^{10}-Methylpteroglutamic acid*	18
Coxiella burnetii	13, 16, 20
Folic acid	23
Pteropterin	28

* Compounds chromatographed on different columns and placed in proper sequence. Eluates assayed by *L. casei* as described in text.

modified to the extent that 15-cm columns were employed, and 10 to 15 ml of sample extract were adsorbed on the column and followed by two washings with 10 ml of 0.2% ascorbate (pH 6.0).

Reference samples of folic acid, citrovorum factor, and pteropterin were obtained from the National Institutes of Health laboratories; tetrahydrofolic acid was prepared by Fred Jones of the University of Kansas. All other folic acid forms were gifts from Phoebe M. Luttner, of G. Toennies' laboratory.

Column eluates were assayed for folic acid activities with *Pediococcus cerevisiae*, *Lactobacillus casei*, and *Streptococcus faecalis* R (Silverman et al., 1961; Mattheis, 1962). Microbial growth response was measured in a Bausch & Lomb Spectronic-20 colorimeter at 650 mμ.

Samples were oxidized in the cold by bubbling O_2 through the solution for 1 hr. Rickettsial eluates and a known folic acid sample were identically formylated by the methods of Gordon et al. (1948). Samples were reduced in a microhydrogenator in the presence of a PtO catalyst (O'Dell et al., 1947). Conjugases were prepared from hog kidney (Bird et al., 1945, 1946) and from chicken pancreas (Mims and Laskowski, 1945).

RESULTS

A number of folic acid forms and sample extracts were chromatographed on DEAE-cellulose columns; Table 1 presents the order of elution. DEAE columns prepared at different times resulted in slightly different elution positions but the relative positions and elution profiles remained constant.

Myers (1958) found that *C. burnetii*-infected chick embryo tissue contained less total folic acid than corresponding uninfected tissue, so a more detailed and critical examination was made of infected and uninfected developing chick embryos (Table 2). The lower folic acid content of infected tissues confirms Myers' observations. Since folate is extracted from *C. burnetii* by our procedures, it cannot be assumed that the folate decrease in infected tissue is due to removal of folate and storage by *C. burnetii*. It is of further interest that both infected and uninfected embryo tissues have elution peaks at tube 16, which increase in folate concentrations as the embryo grows older. There is no folate in tube 16 for either yolk or yolk-sac material of normal or infected eggs. Analyses of albumen showed no detectable folate. Studies were made of folic acid in yolk sacs of uninfected eggs during the purification procedures. After the first Celite treatment, there were 3.5 mμg of folate per mg of acetone-powder preparations of yolk-sac homogenates; after complete purification procedures, no folate was detected.

TABLE 2. *Folic acid content of uninfected and infected chick embryo tissues**

Tissue	Age	Folic acid (mμg per mg N of powder)					
		Uninfected			Infected		
		Tube 11	Tube 16	Total	Tube 11	Tube 16	Total
	days						
Yolk	0	86.9	0	86.9			
	6	84.3	0	84.3			
	10	58.5	0	58.5	29.4	0	29.4
	15	44.6	0	44.6	38.5	0	38.5
Yolk sac	6	34.1	0	34.1			
	10	42.1	0	42.1	45.9	0	45.9
	15	40.9	0	40.9	9.5	0	9.5
Embryo	6	5.49	2.51	8.00			
	10	5.70	10.8	16.5	7.81	3.60	11.4
	15	37.5	64.1	91.6	12.2	20.3	32.5

* Fractions assayed by *L. casei* as described in text.

Comparisons of the folic acid elution profiles of *C. burnetii* and uninfected egg tissues are shown in Fig. 1. The patterns of the uninfected tissue are fairly similar, with peaks at positions 11 to 13 for both rickettsiae and egg tissue. Of interest is the peak at tube 20 in the *C. burnetii* pattern, which is either absent or below detectable levels in egg tissue. The tube 20 eluates were saved for subsequent studies. Several different batches of purified *C. burnetii* powders were extracted for folic acid analysis. Table 3 shows the folate content in tubes 11, 16, and 20, and the wide range of folate concentrations. Occasionally, no folate was detected in the tube 11 position, but tube 20, the position unique to *C. burnetii* folate, consistently contained the folic acid form. It is of additional interest to note that, while the tube 16 eluates are absent in the infected and uninfected yolk and yolk sacs, they are present in the embryo as well as the rickettsiae.

Some characterizations of the unique rickettsial folate (RF) were attempted. Oxidation resulted in a form which was maximally eluted one or two tubes earlier and which was resistant to subsequent additional oxidation. When RF was formylated and chromatographed, no detectable folate could be recovered, although formylation of standard folic acid under identical conditions did yield recoverable folate. Reduction of RF gave an elution pattern with a peak eight tubes earlier than unreduced RF, as well as an additional peak at the usual position.

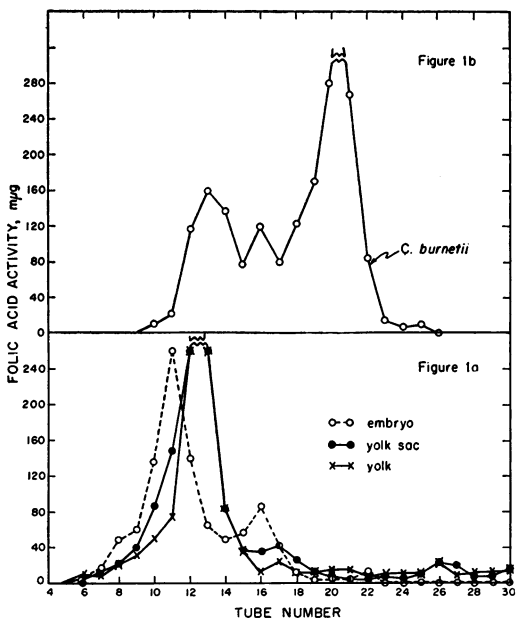

FIG. 1. *Folic acid elution patterns of Coxiella burnetii and uninfected host tissue.* (1a) *Acetone powders of: yolk, 150 mg; yolk sac, 230 mg; embryo, 128 mg.* (1b) *Acetone powder of C. burnetii, 30.6 mg. Chromatography conducted on DEAE columns as described in text; fractions assayed by L. casei as described in text.*

TABLE 3. *Folic acids of Coxiella burnetii**

Sample	Acetone powder	LD_{50}	Folic acid per mg of powder			Total folic acid per mg of powder
			Tube no.			
			11	16	20	
	mg			*mμg*		*mμg*
1	39.1	6.5	4.35	5.63	>6.4	>16.4
2	50.0	6.4	0	0.4	4.12	4.52
3	50.0	6.3	0.68	1.94	3.46	6.08
4	52.2	6.4	0	0.56	2.22	2.78
5	59.5	6.55	0	0.13	0.08	0.21
6	120.4	6.4	3.60	>5	3.07	>11.7
7	294.5	6.55	0.08	0.61	0.68	1.37

* The LD_{50} values are of purified *C. burnetii* suspensions before acetone treatment. Samples 1 and 3 were purified in 0.25 M sucrose diluents; all others in 0.7 M sucrose. Powders extracted and eluted on DEAE-cellulose; fractions assayed by *L. casei* as described in text.

Doctor and Couch (1953) showed that chicken-liver folates are mainly polyglutamates, which on degradation to monoglutamates by conjugase give a growth response for *S. faecalis* R, the assay organism responding only to monoglutamates. Accordingly, a 76-mg sample of *C. burnetii* powder was extracted. It contained 3.4 mμg of folate per mg of powder by *L. casei* assay, but gave no growth response for *S. faecalis* R or *P. cerevisiae*. After treatment with conjugase and correction for endogenous conjugase folate, the powder gave the following responses for all three assay organisms, expressed as mμg folate per mg of powder: *L. casei*, 8.2; *S. faecalis* R, 6.3; *P. cerevisiae*, 2.5. Under these conditions the peak of the treated and chromatographed sample was five tubes previous to the original RF peak, the latter virtually disappearing on conjugase treatment. The presence is indicated of either a polyglutamate or a folic form available only to *L. casei*, similar to prefolic A. The latter, a

methyl derivative of tetrahydrofolic acid, has been shown by Silverman et al. (1961) to be active only for *L. casei*.

DISCUSSION

Folic acid analyses of infected and uninfected chick embryos show in all cases a lower folate content of infected tissue, in agreement with Myers (1958). The lower concentrations are not due to incorporation of folate by the rickettsiae, as claimed by Kleinschmidt et al. (1956), since our assay techniques demonstrably released folate from the parasites, and the rickettsial folate concentrations were negligible compared with host folate. Although rickettsial folate is low, it is of the same magnitude as bacterial folic acid (Long, 1961). Furthermore, a type of folate, either unique to *C. burnetii*, or of very low concentration in host tissues, is present in the rickettsia. This finding, together with the consistent demonstration in *C. burnetii* of a folic acid form present in chick embryo but not in yolk or yolk sac, is suggestive of folate participation in rickettsial metabolism. This is consistent with the demonstration of hydroxymethylation in *C. burnetii* (Myers and Paretsky, 1961). The very large peak of rickettsial folate indicates either a transformation of host folate by the rickettsiae, or a concentration within the rickettsiae if this form of folate is present in host tissue in concentrations too low to have been detected. In any case, a role of folate in rickettsial biochemistry seems indicated. Silverman et al. (1961) had shown an analogous situation: N^{10}-formyltetrahydrofolic was the predominant form of folic acid in mouse leukemic cells, while in the host mouse liver it was prefolic A. The folate chromatograms of leukemic and host liver cells were also significantly different.

Infected erythrocytes contained far more folate than uninfected cells, even when plasmodial folate was taken into account (Trager, 1959). The folic acid forms in the plasmodia were unidentified. Trager suggested an alteration in host-cell metabolism because of the malarial parasite, "which itself has a requirement for folinic acid or some closely related substance." Trager (1961) further studied the effects of sulfadiazine on the above systems, concluding that the folinic acid-synthesizing mechanisms are present mainly in the erythrocytes rather than in the parasites. Although the general resistance of rickettsiae to sulfonamides indicates that the organisms probably do not synthesize much of their own folate, depending rather on the host as a folate source in a manner similar to malaria parasites (Trager, 1959), the present experiments with *C. burnetii* indicate some type of capacity for folic acid synthesis by the rickettsia.

Colón and Moulder (1958) and Colón (1962) used paper chromatography-bioautography to demonstrate in the psittacosis group folic acid forms which differed from that of host allantoic fluid or chorioallantoic membranes. *C. burnetii* folate isolated by the more critical column chromatography supports the concept of folic acid form differences between host and parasite. The interesting folate pictures in malaria-infected erythrocytes, in the psittacosis group, in mouse leukemic cells, and now in *C. burnetii* raises questions on the general folate patterns in virtually obligate or obligate parasitic systems, the biochemical significance of parasite-unique folates, and their roles in host-parasite interrelationships.

ACKNOWLEDGMENTS

We (M.S.M. and D.P.) wish to express our indebtedness to our co-author, M.S., who initiated these studies, and whose keen perceptions laid the outlines for future investigations in this problem.

These studies were supported by grants from the National Science Foundation (G 11466R) and the University of Kansas General Research Fund.

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