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Folic Acid Derivatives in the Gas Gland of *Physalia physalis* L.

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Carbon monoxide is a comparative rarity among the biologically generated C_1 compounds. It has been found in large concentration only in the hollow stems of the kelp, *Nereocystis* (Blinks, 1951), and in the float of the Portuguese man-of-war, *Physalia physalis* L. (Wittenberg, 1958, 1960). A glandular structure, the gas gland or pneumadena, formed by localized modification of the inner wall of the float of *Physalia*, produces carbon monoxide, and will do so *in vitro* when supplied with L-serine. Preliminary analyses indicated that the gas gland is very rich in folates.

The present work establishes that *Physalia* gas-gland tissue is indeed the richest in folates of any animal tissue examined, and that the folates are predominantly N^{10}-formyltetrahydro forms. N^{10}-Formyltetrahydrofolates are the known carriers of the formyl group in several biosynthetic sequences. Their very high concentration in a tissue whose specialized function is the production of carbon monoxide suggests that these folates are the precursors of carbon monoxide. If this be so, then carbon monoxide may be formed by the dehydration of a coenzyme-bound formyl fragment.

In addition to the information obtained on the nature of the folates in the gas glands of *Physalia*, the method presented here may serve as a prototype for the analysis of the varieties of folates occurring in other animal tissues.

MATERIALS AND METHODS

Abbreviation. DEAE-cellulose, diethylaminoethylcellulose.

Gas glands. Physalia were collected on the beaches at Miami, Fla., in March. All animals were alive and were dissected within a few minutes after they had been stranded by an on-shore wind. The outer layer of the float (pneumatocodon) was slit and the inner layer (pneumatosaccus) brought to the Laboratory for further dissection of the gas gland. The gas glands were stored frozen in solid carbon dioxide.

The frozen glands were made into acetone-dried powders by suspending 1 g. of the minced tissue in 100 ml. of acetone (−20°) and homogenizing the suspension in a mechanical homogenizer (VirTis). The solid residue was recovered by filtration, washed with 100 ml. of acetone, dried for 1 hr. *in vacuo* and stored at −10°.

Preparation of N^5*-formyltetrahydropteroyltriglutamic acid.* Pteroyltriglutamic acid (diglutamylfolic acid), a gift from Dr E. L. R. Stokstad (Lederle Laboratories), was purified by chromatography. The sample (25 mg. in 2·5 ml.) was adsorbed on DEAE-cellulose and eluted with phosphate buffer, pH 6·0, with a concentration gradient from 0 to 0·5M (Sober & Peterson, 1954; Usdin & Porath, 1957). The triglutamate from the fractions in the major band which possessed the characteristic u.v. spectra (Stokstad, 1954) was recovered by precipitation by the addition of HCl to give a pH of 3·0. The precipitate was recovered by centrifuging, washed with 1 ml. of water, dissolved in 0·1N-KOH, reprecipitated as a gel at pH 3·0, washed with water and dried *in vacuo*. The yield was 8·1 mg., and the product had a molecular extinction coefficient of 23 400 at 257 mμ in 0·1N-KOH.

The synthesis of the N^5-formyl derivative is based on the methods employed by Roth *et al.* (1952) and Flynn, Bond, Bardos & Shive (1951). To 2·0 mg. of the purified pteroyltriglutamic acid, dissolved in a minimal volume of 0·1N-KOH, were added 0·2 ml. of 10% (w/v) potassium ascorbate, pH 6·0, and 13·2 mg. of sodium dithionite, and the reduction was allowed to proceed for 90 min. at 75°. Then 2·7 ml. of 85% (v/v) formic acid was added and the reaction mixture was kept at 75° for 30 min. Solid ascorbic acid (5–10 mg.) was then added to the chilled reaction mixture, after which it was extracted with diethyl ether till almost free of formic acid. Additional ascorbate (0·15 ml. of 1%, w/v; pH 6·0) was added to the reaction mixture and the pH was adjusted to 6·5–7·0 with KOH. The mixture was autoclaved for 45 min. at 18 lb./in.² steam pressure. The product was purified by chromatography on DEAE-cellulose and the peak (three 5 ml. fractions) corresponding to N^5-formyltetrahydropteroyltriglutamic acid contained 0·5 mg. of the spectrophotometrically 'pure' product. The spectra of the three fractions in the range 220–390 mμ were identical with that of N^5-formyltetrahydrofolic acid (Cosulich, Roth, Smith, Hultquist & Parker, 1952). These fractions were employed without further purification.

The chromatographic properties of N^5-formyl- and of N^{10}-formyl-tetrahydropteroyltriglutamic acid are illustrated in Fig. 5.

Preparation of tissue extracts. Portions (10 mg.) of acetone-dried powder, prepared from the frozen gas glands,

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were extracted in 5 ml. of 1% (w/v) ascorbate solution, pH 6·0, at several temperatures and for varying periods of time. The extracts were cooled to 0° and clarified by centrifuging.

Chromatographic fractionation of the folate derivatives. Chilled portions (3 ml.) of the clear extracts were chromatographed on DEAE-cellulose columns with a gradient ascorbate–phosphate eluent as described by Silverman, Law & Kaufman (1961). Under these conditions, the several naturally-occurring labile folate derivatives are not oxidized significantly. The peak concentrations of N^{10}-formyl- and of N^5-formyl-tetrahydropteroylglutamic acid are found in tubes 8 and 12 respectively. Silverman *et al.* (1961) list the elution patterns of a number of other folic acid derivatives and related compounds.

Determination of folic acid activity. The clear extracts, autolysates or eluted fractions, after suitable dilution in 1% (w/v) ascorbate, pH 6·0, were analysed for folic acid growth activities with *Lactobacillus casei*, *Pediococcus cerevisiae* (A.T.C.C. 8081) (*Leuconostoc citrovorum*) or *Streptococcus faecalis* R., with leucovorin (Lederle) [(±)-N^5-formyltetrahydropteroyl-L-glutamic acid] as the standard. A suitable correction was made for the presence of the inactive isomer. Assay conditions that prevented oxidative changes were employed (Silverman *et al.* 1961).

Acid isomerization of N^5*-formyltetrahydropteroylpolyglutamates.* Concentrated solutions of these folate compounds were prepared by extracting 55 mg. of the gas-gland acetone-dried powder with 5·5 ml. of 1% (w/v) ascorbate, pH 6, at 120° for 45 min. The extracts, clarified by centrifuging, together with a single wash (with 5 ml. of water), were chromatographed on DEAE-cellulose (25 ml., wet volume) columns (Silverman *et al.* 1961) in the absence of any additional ascorbate. Fractions (5 ml.) corresponding to the 2 peaks of the formylpolyglutamates (volumes of 50 ml. each) were combined separately, 100 ml. of chilled 95% (v/v) ethanol was added and the inorganic salts were allowed to sediment at 5° for 90 min. The supernatant solutions were further concentrated to 5 ml., 10 ml. of 95% (v/v) ethanol was added and inorganic salts were allowed to sediment further overnight at 5°. The supernatant solutions were concentrated to 1 ml. *in vacuo*.

Portions (0·2 ml.) were acidified by adding 0·22 ml. of N-HCl in the presence of 0·02 ml. of 10% (w/v) ascorbate, pH 6. The N^5-formyl derivatives were isomerized to the N^{10}-formyl derivatives by heating the reaction mixtures at 100° for 5 min. (Rabinowitz, 1960), and the derivatives were characterized by DEAE-cellulose chromatography after adjustment to pH 6 with 0·5N-KOH in the presence of 1 ml. of 0·2% ascorbate, pH 6.

RESULTS

Folic acid activity of the gas gland of Physalia. Wittenberg (1960) pointed out that the gas gland contains high concentrations of folic acid derivatives. An examination of acetone-dried powders of these glands indicated total folate values (*L. casei* activities) ranging from 220 to 270 μmg./mg. The values shown in Table 1 depend in part on the nature of the treatment of the sample before analysis. The folates in samples autolysed for 60 min. at 37° are primarily in the form of mono-glutamate derivatives of pteroic acid; the folates in the samples extracted at 120° consist of mixtures of mono- and poly-glutamate derivatives. The *S. faecalis* values represent primarily the sum of the monoglutamates present, whereas those forms measured by *P. cerevisiae* consist largely of mono-glutamate derivatives of folic acid reduced to the tetrahydro level. The *L. casei* activities represent responses to mono- and poly-glutamyl derivatives present (Jones, Guest & Woods, 1961). The variations in the values found with *L. casei* as an assay organism are the consequence of the variable responses of the growth of this organism to the several forms of folate present. With a mono-glutamate as the reference standard, the response of this organism to polyglutamates is aberrant, yielding less than proportional growth at lower concentrations and more than proportional growth at higher concentrations (Johnson, 1946; Bird & Robbins, 1946). Thus the values found in specimens not permitted to autolyse depend on the size of the samples employed in the assay system. For the assessment of the total folate concentration, the more valid values are those obtained after the folates are converted into the mono-glutamates (i.e. after 1 hr. at 37°).

The maximal value for the folate concentration found in acetone-dried powders of gas glands is 270 μmg./mg. Liver is the tissue of domestic animals in which the highest concentrations of this vitamin have been observed. Chang (1953) reported values ranging from 4 to 20 μmg./mg. fresh weight in the livers of several species of animals. Since the acetone-dried powders of the gas gland represent 15% of its wet weight, the folate concentration on a wet-weight basis is about 40 μmg./mg. or approximately twice that of livers, which are considered to be a rich source of the vitamin. Thus far, of all animal tissues examined, the gas gland in *Physalia* represents the tissue richest in total folates.

Nature of folates occurring in the gas gland of

Table 1. *Influence of the duration and temperature of extraction on the folic acid content of the gas gland of* Physalia

Experimental details are given in the text.

Conditions of extraction	Folic acid activity (μmg./mg. of acetone-dried powder)		
	L. casei	*P. cerevisiae*	*S. faecalis* R.
37°, 60 min.	270	180	235
37°, 30 min.	250	160	215
50°, 30 min.	250	130	180
60°, 30 min.	240	115	155
75°, 30 min.	220	79	100
120°, 30 min.	225	100	145
120°, 60 min.	225	110	150

Physalia. The problems faced in the attempt to establish the nature of folic acid compounds as they occur in their native states are illustrated in Figs. 1–4, which represent the chromatograms of extracts of the gas glands of *Physalia* prepared at several temperatures. The patterns obtained depend on the conditions of extraction of the tissue. The conclusion as to which forms occur naturally can only be inferred from an examination of all of the profiles. No valid conclusions can be drawn from a study of the pattern obtained after only a single extraction procedure has been employed.

For example, if the tissue is extracted by incubation for 1 hr. at 37°, the following monoglutamate

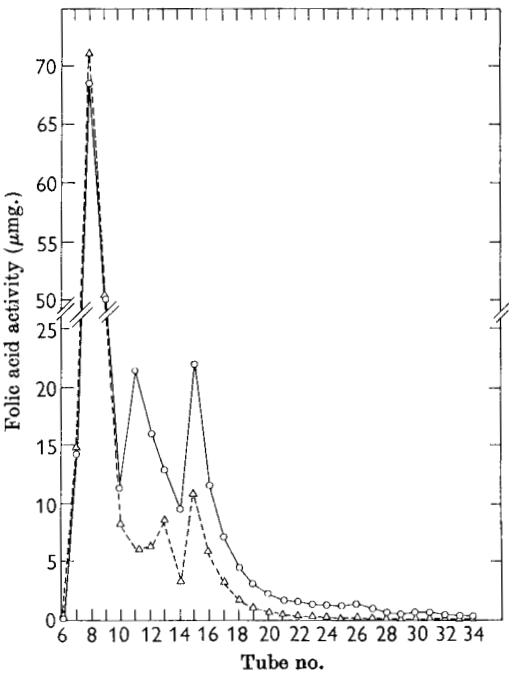

Fig. 1. Folic acid pattern of 1 mg. of acetone-dried powder extracted at 37° for 60 min. ○, *L. casei* activity; △, *P. cerevisiae* activity.

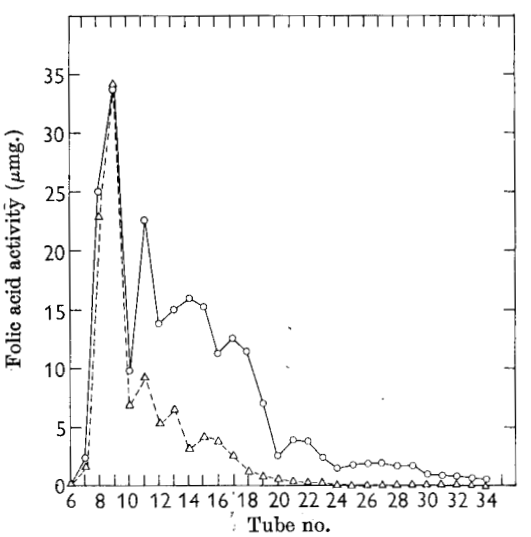

Fig. 2. Folic acid pattern of 1 mg. of acetone-dried powder extracted at 50° for 30 min. ○, *L. casei* activity; △, *P. cerevisiae* activity.

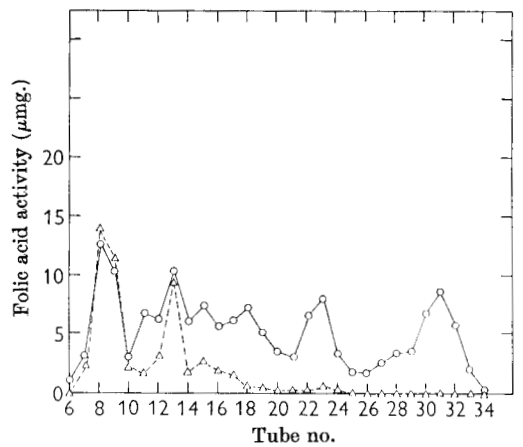

Fig. 3. Folic acid pattern of 1 mg. of acetone-dried powder extracted at 90° for 30 min. ○, *L. casei* activity; △, *P. cerevisiae* activity.

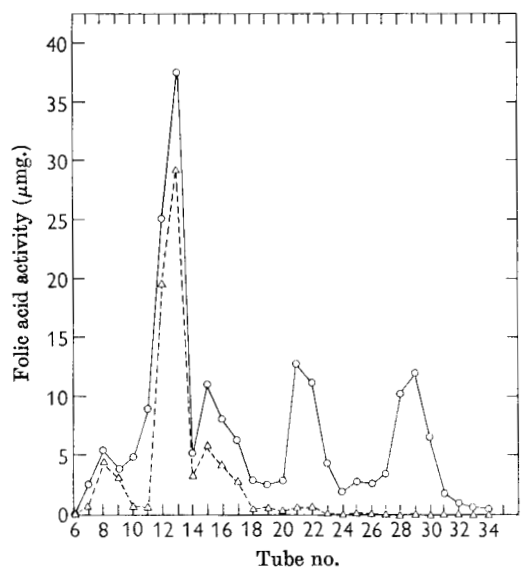

Fig. 4. Folic acid pattern of 1 mg. of acetone-dried powder extracted at 120° for 60 min. ○, *L. casei* activity; △, *P. cerevisiae* activity.

Table 2. *Effect of the temperature and duration of extraction on the relative concentrations of folate derivatives*

Experimental details are described in the text. The Table includes information given in Figs. 1–4. The significance of the scale 0–4 is given in the text.
+, Growth activity corresponding to 50% of that of (−)-N^5-formyltetrahydrofolic acid.

	Tetrahydrofolic acid derivative							
Tube no.	N^{10}-Formyl 8–9	N^{10}-Formyl (oxidized) 11	N^5-Formyl 13	N^{10}-Formyl, diglutamyl 14	Unsubstituted (tetrahydrofolic acid?) 15	N^{10}-Formyl, polyglutamyl 18	N^5-Formyl, diglutamyl 22	N^5-Formyl, polyglutamyl 28–30
Conditions of extraction								
37°, 60 min. (see Fig. 1)	4	4	1	0 (?)	4	0	0	0
37°, 30 min.	4	4	1	0 (?)	4	(1)?	0	0
50°, 30 min. (see Fig. 2)	3	4	1	4	2	4	1	1 (?)
75°, 30 min.	2	2	1–2	2	2	4	2	2
90°, 30 min. (see Fig. 3)	2	2	2	2	1	2	3	3
120°, 30 min.	2	1	3	0 (?)	2	0 (?)	4	4
120°, 60 min. (see Fig. 4)	1	1	4	0 (?)	2	0 (?)	4	4
Growth activity								
For *L. casei*	+	+	+	+	*	+	+	+
For *P. cerevisiae*	+	.	+	.	+	.	.	.
For *S. faecalis* R.	+	+	+	.	+	.	.	.

* Tetrahydrofolic acid is approx. 40–50% as effective as N^5-formyltetrahydrofolic acid (Bakerman, 1961) in supporting the growth of *P. cerevisiae*; for *L. casei*, tetrahydrofolic acid is about 80% as effective as the N^5-formyl derivative.

derivatives of folic acid are found: N^{10}-formyltetrahydrofolic acid at tube 8, N^{10}-formylfolic acid at tube 11, N^5-formyltetrahydrofolic acid at tube 13 and unsubstituted tetrahydrofolic acid at tube 15 (Fig. 1). If, however, the most rigorous extraction procedure is employed, in which the acetone-dried powder suspended in ascorbate solution is autoclaved for 1 hr. at 120°, evidence (Fig. 4) for the following folates is found: a small amount of N^{10}-formyltetrahydrofolic acid (tube 8), N^5-formyltetrahydrofolic acid (tube 13), unsubstituted tetrahydrofolic acid (tube 15), N^5-formyltetrahydropteroyltriglutamic acid (tubes 21 and 22) and what appears to be yet another N^5-formyl derivative of a tetrahydropteroylpolyglutamate (tube 29).

The values shown in Table 2 indicate grossly the occurrence of the several folate derivatives as they appear in the extracts employed in chromatography (Figs. 1–4). The concentration of each specific component is indicated on a scale of 0 to 4, 4 representing the highest concentration of the component encountered. Thus only the values within a column should be compared. The ability of the compounds to support the growth of the test organisms is shown at the foot of the Table. Several trends become apparent from a study of Figs. 1–4 and Table 2. At lower temperatures (under 50°) the predominating formyl derivative is N^{10}-formyltetrahydrofolic acid. Under these conditions, the occurrence of N^{10}-formyltetrahydrofolic acid present as such is measured, as well as that derived from N^{10}-formyltetrahydropteroylpolyglutamate derivatives after the action of endogenous γ-glutamyl peptidase(s) [also known as conjugase(s)]. In other studies the peptidase activity of the gas-gland extract was found to degrade polyglutamylfolates (extracted from chicken liver) stepwise to monoglutamyl end products without significant alterations of the C_1 fragments associated with the naturally-occurring conjugated forms. As the temperature of extraction is increased, peptidase activity is inhibited, permitting an observation of the occurrence of N^5- and N^{10}-formyltetrahydropteroylpolyglutamate derivatives. As the temperature of extraction is increased from 75° to 120°, there is an evident increase in the concentrations of N^5-formyl derivatives at the expense of their N^{10}-formyl counterparts. The latter effect can be explained as a result of the heat-accelerated isomerization of N^{10}-formyltetrahydro to N^5-formyltetrahydro derivatives (May *et al.* 1951).

Evidence for the identity of the formyltetrahydrofolates. The material which emerges at tubes 8 and 9 has been shown to be N^{10}-formyltetrahydrofolic acid. This proof has been presented previously for material derived from another source (Silverman *et al.* 1961). Briefly recapitulated, this folate is eluted in this region (tubes 8 and 9), possesses

essentially equivalent growth activity for the three test organisms, *L. casei*, *P. cerevisiae* and *S. faecalis*, and after heating at 120° is converted into N^5-formyltetrahydrofolic acid (tube 13), which possesses identical microbiological properties.

The evidence that the folates occurring in the regions of tubes 14 and 22 represent N^{10}-formyl- and N^5-formyl-tetrahydropteroyltriglutamates respectively is similar to the above. The elution patterns of authentic N^5-formyltetrahydropteroyltriglutamic acid and its acid isomerization product, N^{10}-formyltetrahydropteroyltriglutamic acid, are shown in Fig. 5. Essentially the same pattern is observed with the material from tube 22 obtained from the extracts of the gas gland (Fig. 6). The biological properties of the synthetic and natural materials are the same in that the *S. faecalis* and *P. cerevisiae* activities represent less than 10 % of that found with *L. casei*. Further, after treatment of the component from tube 22 with pancreatic γ-glutamyl peptidase (Mims & Laskowski, 1945), which converts polyglutamates into diglutamates (Kazenko & Laskowski, 1947), a significant increase in growth activity occurs for the three test organisms (Table 3). The increased growth responses are consistent with the occurrence of a formylpteroylpolyglutamate rather than the methyl analogue, which does not support growth of

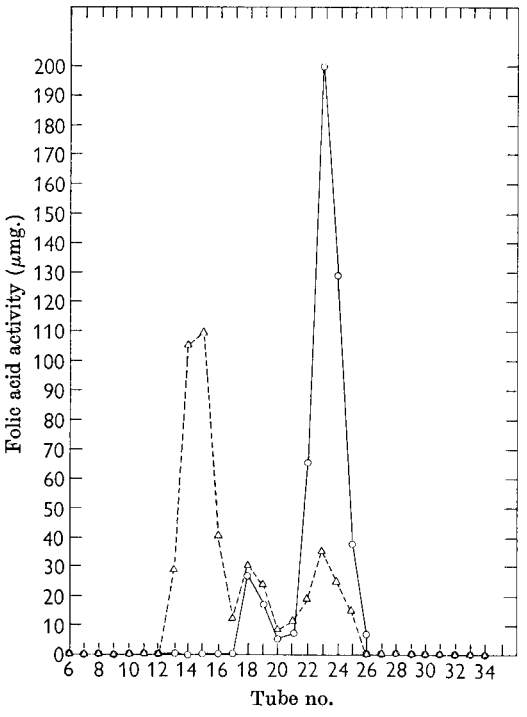

Fig. 5. Comparative chromatograms of synthetic N^5-formyltetrahydropteroyltriglutamic acid (0·5 μg.) before (○) and after (△) acid isomerization.

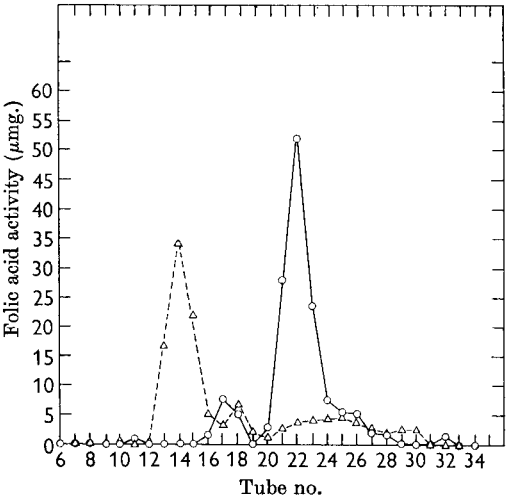

Fig. 6. Comparative chromatograms of active material from tube 22 before (○) and after (△) acid isomerization.

Table 3. *Release of biological activities by chick-pancreas γ-glutamyl peptidase from fractions of extracts of the gas glands of* Physalia

The samples digested were fractions eluted from DEAE-cellulose columns in which the extract of 55 mg. of acetone-dried powder was chromatographed to obtain fraction 22 and an extract of 100 mg. of acetone-dried powder chromatographed to obtain fractions 28 and 29. The procedure of Mims & Laskowski (1945) was employed with the autolysed chick-pancreas γ-glutamyl peptidase preparation without further purification. Values shown are corrected for the small amount of folic acid activity contained in these preparations, and are expressed as μmg./ml.

	L. casei		*S. faecalis* R.		*P. cerevisiae*	
Enzyme treatment ...	Before	After	Before	After	Before	After
Fraction 22						
Sample 1	93	147	<12	88	<12	60
Sample 2	78	120	< 9	81	< 9	69
Fraction 28	185	210	<15	120	<15	120
Fraction 29	175	315	<21	231	<21	189

S. faecalis and *P. cerevisiae* (Larrabee & Buchanan, 1961; Keresztesy & Donaldson, 1961).

The nature of the materials occurring at tubes 18 and 28–30 is as yet not completely established. It can, however, be tentatively concluded that these folates are N^{10}-formyl and N^{5}-formyl derivatives of a pteroylpolyglutamate. The scarcity of the source (the gas gland of *Physalia*) does not readily permit isolation of the folate in amounts required to characterize it by conventional methods. However, a folate with the identical biological and migrational properties of the component eluting at tubes 28–30 is present in, and has been purified from, the bacterium, *Pediococcus cerevisiae* (H. Bakerman & M. Silverman, unpublished work). This folate from *P. cerevisiae* possesses an ultraviolet spectrum identical with that of N^{5}-formyltetrahydrofolic acid and has at least 3 glutamic acid residues/molecule of pterin. When heated for 5 min. at 100° in 0·1 N-hydrochloric acid, the bacterial product shows a shift in the ultraviolet-absorption maximum from 285 to 355 mμ. The latter represents the changes associated with an isomerization of a N^{5}-formyl- to a N^{10}-formyltetrahydrofolate (May *et al.* 1951). The material from *Physalia* emerging at tubes 28–30 behaves in a similar fashion in that, on treatment with acid, it is isomerized to a component which is eluted at tubes 17 and 18 (Fig. 7), the location of the N^{10}-formyl derivative of the folate from *P. cerevisiae*. Further evidence for the polyglutamyl nature of the derivative is derived from the activity of pancreatic γ-glutamyl peptidase. As shown in Table 3, after treatment with γ-glutamyl peptidase significant biological activity is released for *S. faecalis* and *P. cerevisiae*.

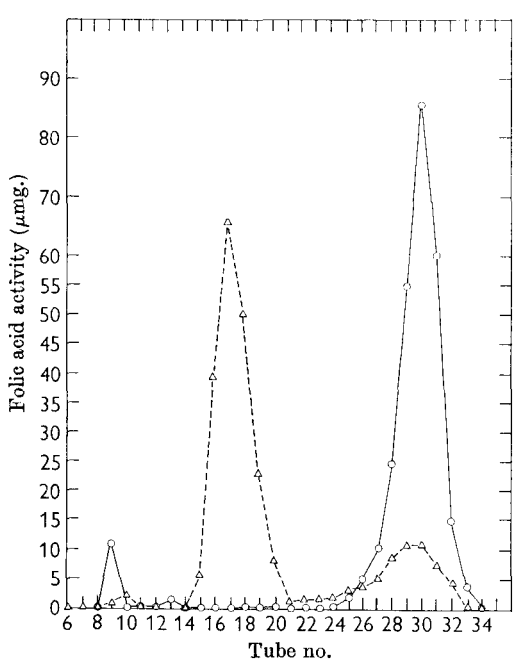

Fig. 7. Comparative chromatograms of active material from tubes 28–30 before (○) and after (△) acid isomerization.

DISCUSSION

Three lines of evidence, namely (*a*) the elution patterns of the folates from DEAE-cellulose, (*b*) the microbiological activity of these extracts and eluted fractions and (*c*) the biological activity of these fractions after treatment with pancreatic γ-glutamyl peptidase preparations, have been used in an attempt to characterize the forms of folate which occur in the gas glands. Taken individually, the elution patterns and the biological activities lead to little understanding of the nature of the folates present. If, however, the collected observations are considered, valid conclusions may be derived. It is readily apparent that interchanges occur at the temperature extremes employed in extraction. At lower temperatures (37–50°), the accumulation of N^{10}-formyltetrahydrofolate represents a summation of this formyl derivative present in the tissue as such and that derived from N^{10}-formyltetrahydropteroylpolyglutamates by the action of endogenous γ-glutamyl peptidase. At the higher temperatures of extraction, the accumulation of the N^{5}-formyl derivatives results from the heat-isomerization of their N^{10}-formyl analogues (May *et al.* 1951) initially present. From these observations it is finally concluded that the folates of the gas gland consist of the N^{10}-formyl derivatives of tetrahydrofolic acid, of tetrahydropteroyltriglutamic acid and of an as yet uncharacterized polyglutamyl derivative of folic acid. The occurrence of N^{10}-formylfolic acid is tentatively considered to be an artifact arising from the non-enzymic oxidation of N^{10}-formyltetrahydrofolic acid. The chromatograms also suggest the presence of unsubstituted tetrahydrofolic acid.

Evidence for the occurrence of glutamic acid conjugates in many natural materials has been presented (Rabinowitz, 1960). The significance of the occurrence of both monoglutamyl and polyglutamyl forms in *Physalia* is obscure. However, in studies with bacterial systems (Wright, 1958; Rabinowitz & Himes, 1960; Jones *et al.* 1961) and liver systems (Blakley, 1957; Jaenicke & Brode, 1961), polyglutamyl folates may be as effective as or more effective than monoglutamates as cofactors in enzymic reactions.

In the light of the observations (*a*) that serine is an effective substrate for carbon monoxide forma-

tion, (*b*) that the β-carbon atom of serine is a precursor of the N^{10}-formyl carbon atom of tetrahydrofolates (Jaenicke, 1955; Deodhar, Sakami & Stevens, 1955) and (*c*) that the gas gland, whose special function is the evolution of carbon monoxide, contains the highest concentration of folates of any animal tissue examined, it would appear probable that tetrahydrofolates are carriers of the C_1 units which ultimately appear as carbon monoxide.

SUMMARY

1. A general procedure is presented for the determination of the several forms of folates present in tissue. Portions of acetone-dried tissue are extracted at several temperatures, the extracts are fractionated on diethylaminoethylcellulose columns, and the fractions obtained are examined for their ability to support growth of two microorganisms. The nature of the folates originally present is deduced from the collected observations.

2. The folates occurring in the gas glands of the Portuguese man-of-war, *Physalia physalis* L., are shown to be primarily N^{10}-formyl derivatives of tetrahydrofolic acid and of tetrahydropteroyltriglutamic acid, and of an as yet uncharacterized polyglutamyl derivative of folic acid.

3. It appears probable that the N^{10}-formyl derivatives are carriers of the C_1 units which are ultimately secreted as carbon monoxide by the gas gland.

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