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295 - Preparation of Tetrahydrofolic Acid and Related Compounds by Electrochemical Methods

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Summary

An electrochemical synthesis of 5, 6, 7, 8–tetrahydrofolic acid (H_4PteGlu) from folic acid (PteGlu) has been developed. In the first step folic acid is reduced, via 5,8–dihydrofolic acid (5,8–H_2PteGlu), to 7,8–H_2PteGlu. Reduction of the protonated 7,8–H_2PteGlu cleaves C(9)–N(10), but reduction of the unprotonated compound at a more negative potential leads to H_4PteGlu in high yield. The reduction is made at relatively low buffer concentration which permits having a neutral pH in the bulk of the solution, but a high pH at the electrode surface due to the electrogenerated base.

Besides the preparation of H_4PteGlu, the reduction of 5,10–methylidyne–H_4PteGlu and 5,10–methylene–H_4PteGlu was studied.

Introduction

A polarographic investigation[1,2] of folic acid suggested that folic acid was reduced in the first step via 5,8–H_2PteGlu to 7,8–H_2PteGlu; in neutral or acidic solution at a more negative potential the protonated 7,8–H_2PteGlu was reduced to 6–methyl–7,8–dihydropterin (6–methyl–H_2Pte), which at a slightly more negative potential was further reduced to 6–methyl–5,6,7,8–tetrahydropterin (6–methyl–H_4Pte). At higher pH a reduction of 7,8–H_2PteGlu to H_4PteGlu was proposed. A medium of high pH has certain disadvantages such as hydrolysis of the compounds and a slow tautomerization of 5,8–H_2PteGlu to 7,8–H_2PteGlu.

A further investigation of these reactions could be of interest for several reasons; a convenient synthesis of H_4PteGlu from folic acid might be developed, and there might be found means (*e.g.* chiral electrodes[3]) to influence the distribution of the stereoisomers of H_4PteGlu in a desired direction. It could possibly also be an attractive way for the preparation of 6–methylpterin.

Of analytical interest might be the electrochemical cleavage of the C(9)–N(10) bond, which occurs under very mild conditions in the reduction of 7,8–H_2PteGlu under slightly acidic to neutral conditions. Since H_4PteGlu is easily oxidized to 7,8–H_2PteGlu a general method for the analysis of the polyglutamyl side chain of H_4PteGlu might be developed.

Below is reported an investigation of the electrochemical reduction of pteroylglutamic acid, of 5,10–methylidyne–H_4PteGlu and of 5,10–methylene–H_4PteGlu. The reaction mechanism was investigated by means of classical polarography, cyclic voltammetry and controlled potential reduction.

Experimental

Instrumentation

The polarographic measurements were carried out on a RADIOMETER POLARITER PO4; the potential control was made with a transistorized potentiostat (TAGE JUHL ELECTRONICS, Copenhagen), and the amount of electricity consumed was measured with an electromechanical integrator. Pulse polarographic and cyclic voltammetric studies were performed on a PRINCETON APPLIED RESEARCH model 174 polarographic analyzer and model 175 universal programmer, together with a WATANABE WX441 XY–recorder. The cells and electrodes have already been described.[4] The hanging mercury drop electrode assembly, METROHM BM–05, was used in cyclic voltammetric studies together with an Ag|AgCl electrode as a reference electrode and an Ag wire as the counter–electrode. In the preparative electrolyses the following cathodes were used: a mercury pool electrode, a mercury plated Cu electrode or a glassy carbon plate electrode. The mercury plated copper electrode was prepared as follows: a thin copper plate was immersed in a $Hg(NO_3)_2$ solution until the whole surface was covered with mercury.

For spectroscopic mesurements a PYE UNICAM SP 1800 spectrophotometer was used.

Materials

From SIGMA CHEMICAL Co. were purchased: L–pteroyl glutamic acid, tetrahydrofolic acid and 5–methyltetrahydrofolic acid. Cellulose DE 52 and CM 52 were supplied by WHATMAN.

Electrolyses

All reductions were carried out at one of the cathodes described above. The anode was a carbon rod. All potentials were referred to an Ag|AgCl saturated KCl electrode. The amounts of electrolized material in the different experiments varied between 5–200 mg in a volume of 40–60 cm^3 of buffer with KCl as the supporting electrolyte. The rest of the cell contained aqueous KCl. During electrolysis the cell was wrapped

in Al foil to protect it against light, the temperature kept at 0 °C and N_2 was flushed through continuously. The presence of the different products, generated during the electrolysis, was followed at a D.M.E., directly immersed in the catholyte. Before, during and after electrolysis samples were taken out for spectroscopic analysis. The general procedure was as follows:

Preparation of H_4PteGlu. – Folic acid was reduced at 0 °C in an 0.1 *M* phosphate buffer, pH 7.0, and 1 *M* KCl at –1.8 V. The end of the reduction was determined by polarography and spectroscopy, indicating a 100 % conversion to H_4PteGlu. Mercaptoethanol or ascorbic acid was then added to give a concentration of approximately 0.5 *M*. The catholyte was then transferred under N_2–pressure to a flask containing dry ice and stored at –20 °C. A H_4PteGlu solution containing no antioxidants was prepared as follows: after completion of the reduction the catholyte was injected with a syringe under N_2 pressure directly from the electrolysis cell into a serum bottle through the rubber cap. Before introducing the H_4PteGlu solution the serum bottle had been evacuated through a second syringe needle in the rubber cap and the gaseous phase exchanged several times with N_2. Finally N_2 was flushed through the solution in the flask for 10 minutes before storage at –20 °C. The solution had λ_{max} 298 nm at pH 7.0, $\varepsilon = 28 \times 10^3\ M^{-1}\,cm^{-1}$. In a similar way reductions were also carried out at pH 8.0, 8.5 and 9.0 (0.1 *M* phosphate buffer) at –1.9 V and in an 0.1 *M* Tris–buffer pH 7.0 at —1.8 V.

Preparation of 5,8–H_2PteGlu. – Folic acid was reduced in a 0.1 *M* borate–buffer, pH 8.5, at –1.0 V at 0 °C. The reaction, wich was observed directly by means of a D.M.E., was noted by the appearance of an anodic wave at –0.75 V for 5,8–H_2PteGlu. At the same time samples were taken out for UV spectroscopy. The sample was transferred under N_2 to a micro–quartz–cuvette, lightpathway 0.2 cm. λ_{max} 288 nm ($25.6 \times 10^3\ M^{-1}\,cm^{-1}$) and 335 nm at pH 8.5. For 7–methyl–5,8–dihydropterin λ_{max} 243 nm ($15.3 \times 10^3\ M^{-1}\,cm^{-1}$), 280 nm ($8.6 \times 10^3\ M^{-1}\,cm^{-1}$) and 335 nm at pH 9.0.

Preparation of 7,8–H_2PteGlu. – Folic acid was reduced at pH 8.0 at –1.0 V. The same procedure was followed as for the preparation of H_4PteGlu to isolate the H_2PteGlu. Concentrations were determined polarographically or spectrophotometrically. The H_2PteGlu could also be obtained as a solid by precipitating with ascorbic acid and concentrated HCl and recovered by centrifugation. λ_{max} 282 nm ($28 \times 10^3\ M^{-1}\,cm^{-1}$) and 224 nm at pH 7.0. However, the solid H_2PteGlu is rapidly oxidized in contact with air.

Preparation of 5,10–methylidyne H_4PteGlu. – The procedure outlined by HUENNEKENS[5] and by ROWE[6], has been modified slightly. After complete reduction to H_4PteGlu, the catholyte was transferred under N_2 to a round–bottomed flask containing dry ice. The solution was lyophilized immediately and deaerated. Concentrated formic acid was introduced

under N_2 pressure. The mixture was kept at 60 °C for 3 hours while N_2 was flushed through. At this stage the yield of 5,10–methylidyne–H_4PteGlu was 90–98 %, based on the original amount of folic acid, measured by spectrophotometry and using $\varepsilon_{355} = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$. For further purification the solution was applied to a K 62/40 Sephadex column packed with WHATMAN CE 52 cellulose cation–exchanger and eluted with 2.5 *M* formic acid. All fractions having an absorbancy ratio A_{355}/A_{280} greater than 1.6 were collected and evaporated to dryness. The residue was recrystallized from 0.1 *M* HCl. λ_{max} 284 nm and 362 nm ($26 \times 10^3\ M^{-1}\ cm^{-1}$) in 0.01 *M* HCl. λ_{max} at 348 nm ($26.5 \times 10^3\ M^{-1}\ cm^{-1}$) in 0.1 *M* HCl.

Preparation of 5–methyl–H_4PteGlu. – After complete reduction of folic acid to H_4PteGlu a tenfold excess of aqueous formaldehyde (35 %) was added with the formation of 5,10–methylene–H_4PteGlu. An attempt to reduce the latter compound electrochemically to 5–methyl–H_4PteGlu resulted only in very low yields. For preparative purposes reduction was therefore accomplished by the use of $NaBH_4$. The product was purified by column–chromatography on WHATMAN DE 52 cellulose anion–exchanger, following mainly the procedure of GUPTA *et al.*[7] and BLAIR *et al.*[8] λ_{max} 290 nm at pH 7.0 (292 nm).[9]

Oxidation of 5–methyl–H_4PteGlu. – 5–methyl–H_4PteGlu was dissolved in a 0.1 *M* phosphate buffer pH 8.0, previously deaerated with N_2. The compound was oxidized electrochemically at –0.1 V at a platinum electrode with exclusion of air. The resulting quinonoid 5–methyl–H_2PteGlu rearranged very slowly (several days) on standing to another dihydro–H_2PteGlu, which could be converted to the pterine derivative by reductive eavage of the C(9)–N(10) bond. Both 5–methyl–H_2PteGlu isomers could be reduced electrochemically to 5–methyl–H_4PteGlu.

Oxidation of H_4PteGlu. – After complete reduction to H_4PteGlu, the Hg cathode was removed and replaced by a platinum anode. Oxidation was carried out at –0.2 V under N_2 to the 6,7–H_2PteGlu, which rearranged very rapidly to the 7,8–H_2PteGlu. The latter compound could be oxidized by air to the original folic acid. Yield of the complete cycle was 85–100 %.

Reduction of 5,10–methylidyne–H_4PteGlu. – Between 10–30 mg were added to a previously deaerated buffer solution and reduced at a Hg cathode. In a phosphate buffer of pH 6.0, reduction at –1.7 V gave the 5,10–methylidyne–H_4PteGlu ; in an acetate buffer of pH 5.0 at –1.5 V the products were 5,10–methylidyne–H_4PteGlu and a small amount of 5–methyl–H_4PteGlu. In more alkaline solutions (pH 9.0) or reduction by $NaBH_4$ the product was predominantly 5–formyl–H_4PteGlu. The products were identified by UV–spectroscopy.

Preparation of 6–methylpterin and 6–methyl–7,8–dihydropterin. – Folic acid was reduced in a 0.5 *M* borate buffer pH 7.0 at –0.9 V. After complete reduction to 7,8–H_2PteGlu, ascorbic acid was added (0.5 *M*)

and the reduction continued at –1.1 V. After complete conversion to 6–methyl–7,8–H_2PteGlu, the catholyte was acidified and the precipitate collected and purified by dissolving it in 1 *N* NaOH and precipitating by 10 *N* NaOH. λ_{max} 250 and 365 nm at pH 1.0 (250 and 364 nm)[10], λ_{max} 280 and 322 nm in 0.1 *N* NaOH (280 and 326 nm).[10] To obtain the 6–methylpterin, the 7,8–H_2Pte solution was first treated with H_2O_2 before acidifying. The 6–methylpterin was purified in the same way. λ_{max} 247 and 232 nm at pH 1.0 (248 and 324 nm)[10] λ_{max} 253 and 357 nm at pH 13.0 (253 and 365 nm).[10]

Analysis. – To determine if a cleavage of the C(9)–N(10) bond had taken place during reduction *p*–aminobenzoylglutamate was determined in the catholyte according to a modified BRATTON–MARSHALL[11] procedure. Usually between 1–2 % *p*–aminobenzoyl–glutamate was present after reduction, but up to 50 % was found if the reductions were carried out at room temperature. The H_4PteGlu preparations were tried for substrate activity for FIGLU enzymes, following the procedure of TABOR and WIJNGARDEN.[12] Typical values of enzyme activities for different batches of electrochemically prepared H_4PteGlu were from 47 to 52 %. As a comparison a commercial sample of H_4PteGlu showed enzyme activies from 42–46 %, indicating that the product was a mixture of equal amounts of both diastereomers of L–H_4PteGlu. Substrate activity for 5,10–methylene tetrahydrofolate dehydrogenase according to RAMASASTRI and BLAKLEY,[13] showed enzyme activities of 34–36 % for the electrochemical preparation and 39–42 % for the commercial sample.

Cyclic voltammetry. – Cyclic voltammetric studies were conducted on folic acid and its reduced derivatives, either on the isolated compounds or directly in the solution.

Results and discussion

The preparation of H_4PteGlu from folic acid is generally made by reduction with an excess of $NaBH_4$; an electrochemical synthesis seems an attractive alternative, since the work–up would be very simple. However, certain problems had to be solved, before the method could be used.

One problem was that 7,8–H_2PteGlu, an intermediate between PteGlu and H_4PteGlu possesses the electrophor –C(=Y)CHXR, where Y is a heteroatom and X^- a good leaving group. Such an electrophor[14] under suitable conditions is reduced to –C(=Y)CH_2R + X^-, which for 7,8–H_2PteGlu means that the bond C(9)–N(10) is cleaved. This cleavage takes place, when the protonated 7,8–H_2PteGlu is the reduced species.

The N(5)–C(6) azomethine group is, as is the case for all azomethine groups,[15] more easily reducible in the protonated form than in the unprotonated one. Protonation is thus favourable for the uptake of electrons, but it also makes the anilino group a better leaving group. For unprotonated 7,8–H_2PteGlu the leaving group would be a derivative of

aniline anion, which is a strong base, but if N(10) is protonated, the leaving group is the much weaker base, a derivative of aniline. Protonation thus favours the cleavage.

A further complication arises from the fact, that not only the equilibrium concentration of the protonated form in the equilibrium

$$7,8\text{–}H_2PteGlu + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 7,8\text{–}H_2PteGluH^+ \qquad (1)$$

is of interest, but also the rate constant (k_1) of the protonation reaction. The reason is that the more easily reducible, protonated form is removed from the equilibrium by reduction, and at a given pH the reactants will try to reestablish the equilibrium by protonation of 7,8–H_2PteGlu. If k_1 is large, the protonated form may be the a reduced species even some pH–units higher than the pK of the system. Reductions involving such preprotonations are well–known from polarographic investigations.[16]

At pH 8 the equilibrium of (1) is shifted well to the left, but the rate of protonation is not negligible at a high buffer concentration; at pH higher than 8, hydrolysis becomes a complicating factor. The rate of protonation may be lowered by working at low temperatures and using a low concentration of buffer. As seen from the equation

$$RR'C{=}NR'' + 2\,e^- + 2\,H_2O \rightarrow RR'CH\text{–}NHR'' + 2\,OH^- \qquad (2)$$

a base is created close to the electrode, and the electrochemical reduction thus takes place in surroundings which have a higher pH than that measured in the bulk of the solution. At relatively low buffer concentrations the pH–difference between the bulk and the electrode surface during electrolysis is sufficient to ensure, on the one hand that the preprotonation reaction (2) is sufficiently slow, and on the other hand that the bulk pH is sufficiently low to avoid serious problems with hydrolysis.

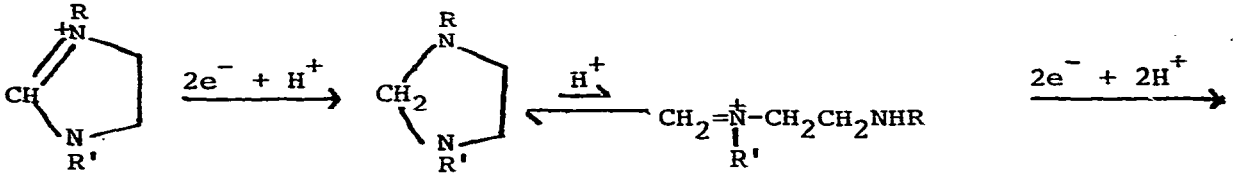
Table 1. Polarographic half–wave potentials of some derivatives of formamidine. V *vs.* S.C.E.; medium containing 40 % alcohol.

pH	N,N′–diphenyl formamidine	N,N′–diphenyl imidazoline	Imidazoline
4.7	—1.33	—1.15	
5.9	—1.35	—1.15	
7.5	—1.42	—1.15	
9.0	—1.55		—1.60
			—1.60

Under such conditions PteGlu can be reduced to H_4PteGlu in good yield; direct UV–determination at 298 nm gave in different runs 85–

100 %, the same yields were found from the recovery of PteGlu after reoxidation. The yield (98–99 %) was also determined from the yield of 5,10–methylidyne–H_4PteGlu (λ_{max} 355 nm) and the concentration (0–1.5 %) of *p*–aminobenzoyl glutamic acid was determined by diazotization (BRATTON–MARSHAL reagent).

5,10–Methylidyne–H_4PteGlu is a cyclic formamidine, an imidazoline, and it is as other formamidines reducible at the dropping mercury electrode (Tab. 1). The primary reduction product from a cyclic formamidine is an imidazolidine, which is not electrochemically reducible as such ; a further reduction requires a ring opening (*e.g.* acid catalyzed) to an azomethine compound, a formimine derivative. The reactions may be formulated



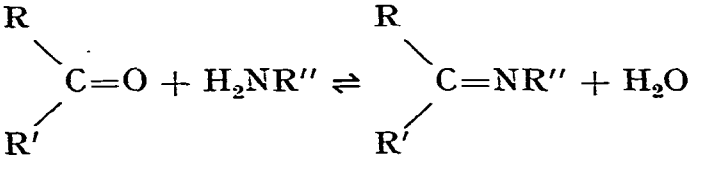
I II III (3)

CH_3–NR′–CH_2CH_2–NHR

IV

The reaction is complicated by hydrolysis of I and III. In the presence of an excess of formic acid I (5,10–methylidyne H_4PteGlu) is under suitable conditions found in a reasonable high concentration in equilibrium with the free diamine, R′NHCH_2CH_2NHR (V), and the formyl derivative, HCONR′CH_2CH_2NHR (VI). Of these (I, V and VI) only I is reducible, so I may thus be reduced electrochemically to II.

The further reduction is more difficult. In an equilibrium consisting of the diamine (V), formaldehyde, hydrated formaldehyde and the formimine derivative (III), III is present in a very low concentration. This would not be an insurmountable difficulty, compare *e.g.* the reductive alkylation of methylamine by cyclohexanone,[17] if the formimino derivative was reduced at a potential sufficiently less negative than that of formaldehyde (4)



(4)

Although a small prewave to the formaldehyde wave, presumably due to the azomethine compound (III), is visible on a polarogram of a

mixture of H_4PteGlu and an excess of formaldehyde, it is too close to the large wave of formaldehyde to permit a selective reduction of III, even though the major part of formaldehyde is present as the unreductible hydrated form.

Although it has been possible to reduce N,N'–diphenylformamidine to the expected mixture of aniline and N–methylaniline, the right conditions for a high yield electrochemical synthesis of 5–methyl–H_4PteGlu from H_4PteGlu and formaldehyde have not yet been found.

5–Methyl–H_4PteGlu can be oxidized electrochemically and the oxidation product reduced again to 5–methyl–H_4PteGlu as evidenced by the quasi–reversible oxidation–reduction couple observed on cyclic voltammetry of 5–methyl–H_4PteGlu at a mercury electrode. Electrochemical oxidation of 5–methyl–H_4PteGlu yields primarily a 5–Me–H_2PteGlu with a quinonoid structure, the 5–Me–6,7–H_2PteGlu. This quinonoid derivative can tautomerize into another isomer, but the tautomerization is much slower than in the case of the 6,7–PteGlu. The reaction is base catalyzed and at pH 7–8 the rate is still very slow.

Several structures have been proposed for the 5–Me–H_2PteGlu[9,18-20] and for the rearranged product,[9,19,21] in analogy with 5–Me–diphenylpterines and lumazines.[22-24]

Several electrochemical data point to 5–methyl–7,8–H_2PteGlu being the electroactive species in slightly acid solution. The absence of an anodic wave seems to exclude an H_4folate derivative with a double bond in the H_2PteGlu derivative in an exocyclic position.[7] The presence of two reduction waves at pH 8 ($U_{½} = -1.18$; $U_{½} = -1.53$) points to

Table 2. Voltammetric results of some folic acid derivatives.
Cathodic (U_{pc}) and anodic (U_{pa}) peak potentials (—V *vs.* S.C.E.) in cyclic voltammetry at a hanging drop mercury electrode, sweep rate 200 mV s^{-1}. Polarographic half–wave potentials ($U_{½}$) at a D.M.E.

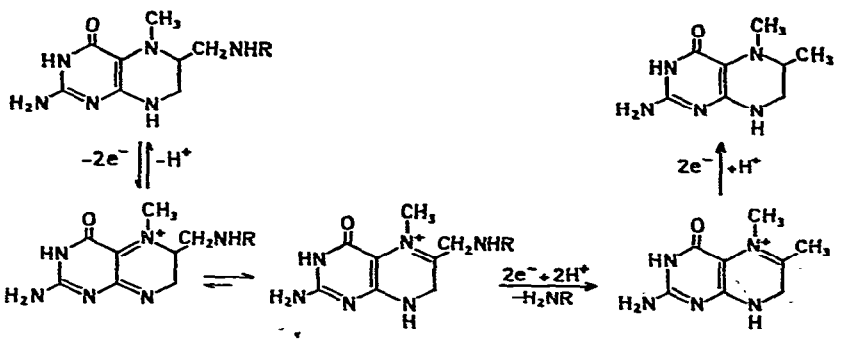
pH	5,10-methylidyne H_4PteGlu	5-Methyl-H_4PteGlu		5-Methyl-6,7-H_2PteGlu		5-Methyl-7,8 H_2PteGlu		
	U_{pc}	U_{pa}	U_{pc}	$U_{½}$	U_{pc}	$U_{½}$ (I)	$U_{½}$ (II)	U_{pc} (I)
6.0	—1.67	—0.17	—0.27					
7.0	—1.69	—0.25	—0.35					—1.18
8.0	—1.72 [—1.17][a]	—0.40	—0.46	—0.35		—1.18[b]	—1.53	—1.20
8.5		—0.42	—0.47		—0.53			—1.26
9.0	—1.75 [—1.19][a]	—0.44	—0.50	—0.40				—1.39
10.0		—0.46	—0.52					—1.35

[a] Small peak on second sweep, possibly due to 5–methylene H_4PteGlu.
[b] After preparative reduction at this potential the catholyte had a wave at –1.55 V.

a reductive cleavage of the C(9)–N(10) bond during the first reduction and a reduction of the thus obtained 5,6–dimethyl–7,8–dihydropterin to the tetrahydropterin.

Preparative reduction at the potential of the first wave yields the 5,6–dimethyl–H_2Pte and aminobenzoylglutamic acid in quantitative yields. A 5,6–dihydroderivative could also produce two reduction waves, where the first one would be a cleavage of a C–N bond and the second one the saturation of an azomethine bond, but in that case the cleavage would involve the C(6)–N(5) bond with a resulting ring–opening. Under strongly alkaline conditions the 5–Me–6,7–H_2PteGlu might partially be converted to a hydroxylated[9,19] or peroxy tetrahydroderivative, followed by rearrangement to an *s*–triazine.[21,23,24] However, these compounds will not yield the 2 cathodic waves. On the other hand the isolated 5–Me–H_2PteGlu, reducible by ascorbic acid, cysteine, homocysteine and mercaptoethanol,[18-20] would presumably be the quinonoid 5–Me–6,7–H_2PteGlu because of the rather positive value of its $U_{1/2}$ and in analogy to 6,7–H_2Pte[25] and 6,7–H_2PteGlu.

On the basis of the above mentioned evidence the following reaction scheme is considered.



Under the assumption that the scheme is essentially correct, further work is in progress along the lines of controlled oxidation of 5–methyl–H_4PteGlu to 5–methyl–H_2PteGlu, followed by reductive cleavage of the C(9)–N(10) bond with the aim of developing a general method for the determination of the side chain in H_4PteGlu derivatives.

References

1 K. Kretzschmar and W. Jaenicke, *Z. Naturforsch. Teil B* **26** (1971) 225
2 K. Kretzschmar and W. Jaenicke, *Z. Naturforsch. Teil B* **26** (1971) 999
3 B.F. Watkins, J.R. Behling, E. Kariv and L.L. Miller, *J. Am. Chem. Soc.* **97** (1975) 3549
4 S. Kwee and H. Lund, *Biochim. Biophys. Acta* **297** (1973) 285

5 F.M. HUENNEKENS, P.P.K. Ho and K.G. SCRIMGEOUR, in *Methods of Enzymology*, S.P. COLOWICK and N.O. KAPLAN (Editors), Academic Press, New York (1963) Vol. VI, pp. 806–811

6 P.B. ROWE, *Anal. Biochem.* **22** (1968) 166

7 V.S. GUPTA and F.M. HUENNEKENS, *Arch. Biochem. Biophys.* **120** (1967) 712

8 J.A. BLAIR and K.J. SAUNDERS, *Anal. Biochem.* **34** (1970) 376

9 J.A. BLAIR, A.J. PEARSON and A.J. ROBBS, *J. Chem. Soc. Perkin Trans. II* (1975) 18

10 R.L. BLAKLEY, *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland Publishing Comp., Amsterdam (1969) p. 67

11 C.A. BRATTON and E.K. MARSHALL JR., *J. Biol. Chem.* **128** (1939) 537

12 H. TABOR and L. WIJNGARDEN, *J. Clin. Invest.* **37** (1958) 824

13 B.V. RAMASASTRI and R.L. BLAKLEY, *J. Biol. Chem.* **237** (1962) 1982

14 L. HORNER and H. LUND, in *Organic Electrochemistry*, M.M. BAIZER (Editor), Marcel Dekker, New York (1973) pp. 731–779

15 H. LUND, in *The Chemistry of the Carbon–Nitrogen Double Bond*, S. PATAI (Editor), Interscience Publishers, London (1973) pp. 505–564

16 J. HEYROVSKY and J. KUTA, *Grundlagen der Polarographie*, Akademie Verlag, Berlin (1965) pp. 307–371

17 H. LUND, *Acta Chem. Scand.* **13** (1959) 249

18 K.O. DONALDSON and J.C. KERESZTEZY, *J. Biol. Chem.* **237** (1962) 3815

19 G.R. GAPSKI, J.M. WHITELEY and F.M. HUENNEKENS, *Biochemistry* **10** (1971) 2930

20 T.L. DEITS, A. RUSSELL, K. FUJII and J.M. WHITELEY, in *Chemistry and Biology of Pteridines*, W. PFLEIDERER (Editor), Walter de Gruyter, Berlin (1975) pp. 525–534

21 J.A. JONGEJAN, H.I.X. MAGER and W. BERENDS, in *Chemistry and Biology of Pteridines*, R.L. KISLIUK and G.M. BROWN (Editors), Elsevier/North-Holland, New York (1978) pp. 241–246

22 M. VISCONTINI and T. OKADA, *Helv. Chim. Acta* **50** (1967) 1492

23 J.A. JONGEJAN, H.I.X. MAGER and W. BERENDS, *Tetrahedron* **31** (1975) 533

24 H.I.X. MAGER, in *Chemistry and Biology of Pteridines*, W. PFLEIDERER (Editor), Walter de Gruyter, Berlin (1975) pp. 753–771

25 S. KWEE and H. LUND, *Bioelectrochem. Bioenerg.* **1** (1974) 87