Volume 278, number 2, 247–251 FEBS 09327 January 1991
© 1991 Federation of European Biochemical Societies 0014-5793/91/$3.50
ADONIS 001457939100108R

Nature of primary product(s) of D-glucose 6-phosphate dehydrogenase reaction

^{13}C and ^{31}P NMR study

Gotam K. Jarori and Pabitra K. Maitra

Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400 005, India

Received 8 October 1990

Glucose 6-phosphate dehydrogenase catalyzes the oxidation of glucose 6-phosphate, resulting in the formation of 6-phosphogluconolactone. As this compound is unstable, it has not been characterized directly. NMR provides a way to directly monitor all components of a reaction and study their structure. Here we report some results on the glucose 6-phosphate dehydrogenase reaction using ^{31}P and ^{13}C-NMR. Our results indicate that two different lactones, namely, γ (1→4) and δ (1→5) 6-phosphogluconolactones, are formed as products in this reaction. This is in contrast to an earlier suggestion that glucose 6-phosphate dehydrogenase produces only the δ-lactone. On the basis of these results, a new mechanism for dehydrogenation of the sugar phosphate is proposed.

D-glucose 6-phosphate dehydrogenase; Lactone; ^{13}C NMR; ^{31}P NMR

1. INTRODUCTION

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP$^+$ oxidoreduction, EC 1.1.1.49) (G6PD) the first enzyme of the pentose phosphate shunt, catalyzes the dehydrogenation of the anomeric carbon (C-1) of glucose 6-phosphate (Glc6p). It has been proposed that G6PD is specific for the β-anomer of Glc6p [1]. Instability of the dehydrogenation reaction product, i.e. 6-phosphogluconolactone, has precluded detailed and unambiguous characterization of the primary product.

Warburg et al. [2] established that the only stable product of this reaction is 6-phosphogluconate. They proposed that dehydrogenation proceeds via a hydrated adduct of the aldehyde group of Glc6p (Scheme I, Fig. 5). Later on it became clear that Glc6p exists in aqueous solution, predominantly in the pyranose form rather than as a straight chain aldehyde. This led Cori and Lippman [3] to propose that the dehydrogenation reaction proceeds directly without the participation of H_2O to yield the unstable δ-lactone as the primary product of oxidation. Here we report some of our experiments where we have studied this reaction using ^{13}C and ^{31}P NMR. Our main objective in performing these experiments was to obtain chemical shift assignments (^{13}C as well as ^{31}P) for the lactone generated in this reaction which can then be used in some whole-cell NMR studies. Interestingly, we observed the formation of two different lactone products in this reaction. This is in contrast to earlier observations. These results and their implications to the reaction mechanism are described below.

Correspondence address G K Jarori, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400 005, India

Abbreviations: G6PD, Glucose-6-phosphate dehydrogenase, Glc6p, Glucose-6-phosphate, 6-PGA, 6 Phosphogluconicacid, 6-PGL, 6-Phosphogluconolactone

2. MATERIALS AND METHODS

2.1 *Chemicals*

Glucose 6-phosphate, NADP, glucose 6-phosphate dehydrogenase (type VII) and baker's yeast hexokinase (type C-300) were obtained from Sigma. 1-[^{13}C]glucose (98.8% ^{13}C) was supplied by MSD Isotopes, Montreal, Canada. Oxidized glutathione (GSSG) and glutathione reductase were from Boehringer-Mannheim. 2-[^{13}C]glucose (99% ^{13}C) was purchased from Sigma. All other chemicals used were of analytical grade. Bakers yeast G6PD from Sigma was passed through a blue sepharose column and eluted with NADP. Fractions containing G6PD were concentrated in an Amicon ultrafiltration cell. This enzyme was used in the experiments. *E. coli* 6-phosphogluconolactonase was prepared from a high copy clone of this enzyme (plasmid kindly given by Dr. Lee Rosner).

2.2 *NMR spectra*

^{31}P (202.5 MHz) and ^{13}C (125.8 MHz) spectra were recorded using Bruker AM-500 instrument equipped with a 10 mm multinuclear probe, a variable temperature controller and an Aspect-3000 computer. ^{31}P chemical shifts have been measured with respect to ~85% H_3PO_4 placed in a capillary as an external reference. ^{13}C shifts are relative to external TSP (sodium trimethyl silyl propionate-2,2,3,3-d_4). All samples contained 20% D_2O for field/frequency lock. All spectra were recorded at room temperature (24 ± 1°C) with ^1H-broad band decoupling.

In the experiment where the dehydrogenase reaction was monitored by ^{31}P-NMR, typical sample composition was: 5 mM Glc6p, 0.1 mM NADP, 1 mM $MgCl_2$, 10 mM oxidized glutathione, glutathione reductase, 40 mM Triethanolamine-HCl, pH 7.0. The reaction was started by addition of G6PD. NADPH produced in the reaction was re-oxidized by glutathione.

In the experiments where ^{13}C-NMR was used to monitor the reaction, 1-[^{13}C] or 2-[^{13}C]glucose (5–6 mM) was first converted to Glc6p by including 10 mM ATP, 10 mM $MgCl_2$ and yeast hexokinase in 40 mM Triethanolamine-HCl, pH 7. This conversion was allowed to proceed overnight at room temperature. Subsequently, glutathione and glutathione reductase were added to the reaction mixture. Time lapse spectra were recorded immediately after the addition of G6PD.

3. RESULTS AND DISCUSSION

Since the substrate as well as product(s) of the G6PD reaction have a phosphoryl group, the reaction catalyzed by this enzyme can easily be monitored using ^{31}P-NMR. Time lapse ^{31}P-NMR spectra were acquired after addition of G6PD to Glc6p. Fig. 1 shows some of these ^{31}P-NMR spectra. Glc6p gives rise to two resonances (peaks 1 and 2 in Fig. 1a) at 4.39 and 4.46 ppm, corresponding to α and β anomers. On starting the reaction by adding G6PD, a new resonance (peak 3, Fig. 1a) at 4.27 ppm is observed. Initially, as the intensity of peak 3 increases, a corresponding decrease in intensities of peaks 1 and 2 (Fig. 1a–d) is observed. In the later part of the reaction, peak 3 could be resolved into two peaks (5 and 6) which are separated by −0.06 ppm. These resonances (peak 3 or peaks 5 and 6) are transient in nature and eventually only one resonance at 4.40 ppm is observable (Fig. 1i).

It is known that G6PD catalyzes the oxidation of Glc6p into 6-phosphogluconolactone (6-PGL). Being unstable, the lactone undergoes spontaneous hydrolysis, resulting in the formation of 6-phosphogluconic acid (6-PGA). Thus peaks 5 and 6, which are transient in nature can be attributed to 6-PGL(s) and the peak at 4.40 ppm to 6-PGA. The identity of peaks 5 and 6 was further confirmed in an experiment (not shown) where purified 6-phosphogluconolactonase (from *E. coli*) was added when considerable resonance intensity was observable for peaks 5 and 6. These two peaks disappeared rapidly with corresponding increase in intensity at peak 4. Addition of 2 mM 6-PGA after spectrum-i (Fig. 1) resulted in increased intensity at peak 4. Thus peak 4 at 4.40 ppm is assignable to 6-PGA and the transient nature of peaks 5 and 6 indicates that they are due to its precursors being convertible to 6-PGA by the action of 6-phosphogluconolactonase.

Since the chemical modification in this reaction occurs at C-1 of Glc6p, we decided to characterize further

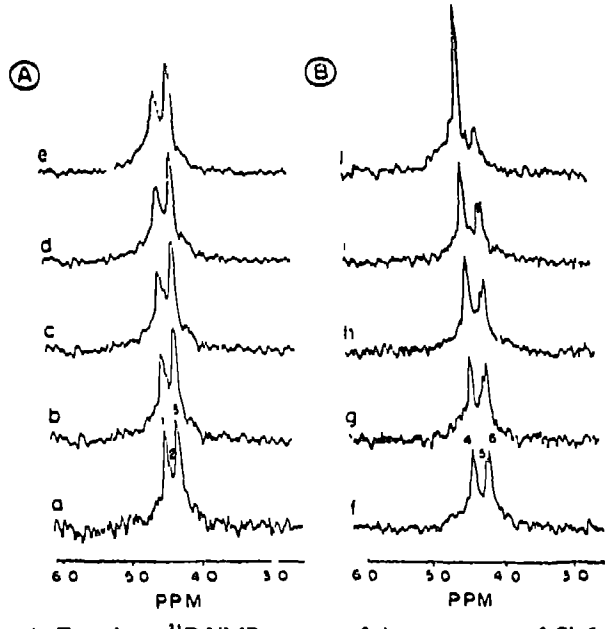

Fig 1 Time-lapse ^{31}P-NMR spectra of the conversion of Glc6p to 6-PGL catalyzed by G6PD. Sample composition is described in section 2. Panel A presents the spectra plotted at various time intervals while data were being acquired continuously. Spectrum (a) is after 32 scans, whereas (b) is after 64 scans. Time lapsed after addition of G6PD at the end of each spectrum in panel A are (a) 10, (b) 14, (c) 18, (d) 29, (e) 35 min. In panel B data were acquired between (f) 40–48, (g) 49–56, (h) 57–64, (i) 65–72, (j) 77–85 min. After spectrum (i), 2 mM 6-PGA was added to the sample and spectrum (j) was recorded.

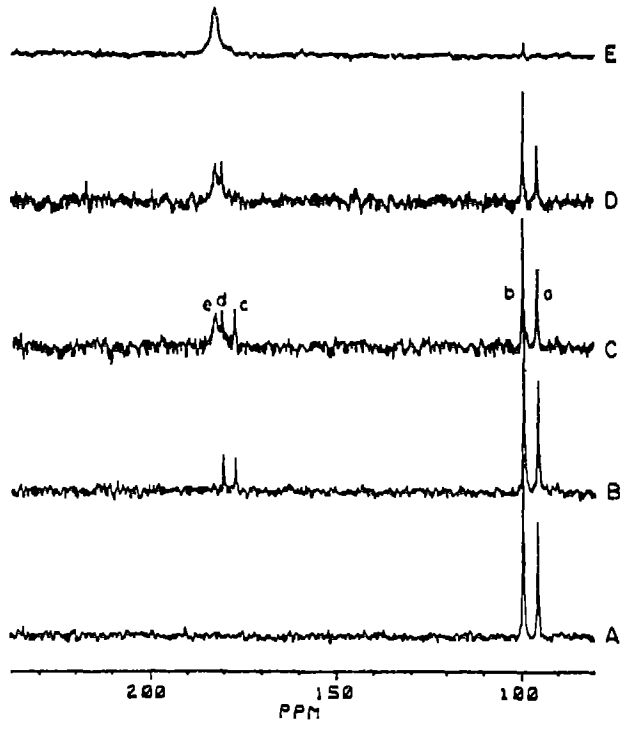

Fig 2 Characterization of products of G6PD reaction by ^{13}C-NMR using 1-[^{13}C]Glc6p. A few relevant spectra acquired at different time intervals after addition of G6PD are shown. Sample composition is given in section 2. Note that various spectra are plotted with different vertical scale expansions for the sake of clarity. Spectrum (A) before adding G6PD, (B) between 0–9, (C) between 18–27, (D) between 27–36 and (E) between 60–69 min after the addition of G6PD. The chemical shifts of various resonances are (a) 95.4, (b) 99.2, (c) 176.9, (d) 180.1 and (e) 182.1 ppm.

the nature of the products by ^{13}C-NMR using 1-^{13}C enriched substrate. 1-[^{13}C]glucose was converted to Glc6p as described in section 2. Time lapse ^{13}C-NMR spectra after addition of G6PD are presented in Fig. 2. Phosphorylation of glucose at C-6, does not affect the chemical shifts of C-1. Resonances observed at 95.2 and 99.1 ppm (peaks a and b, Fig. 2) are from α and β anomers of Glc6p (and/or glucose). Addition of G6PD results in the appearance of two more resonances at 176.9 and 180.1 ppm (peaks c and d in Fig. 2B). With passage of time, these two transient resonances (c and d) disappear and eventually all ^{13}C label appears at 182.1 ppm (peak e in Fig. 2C). In this experiment trace amounts of $MnCl_2$ were included in the sample, in order to identify the resonance due to -COOH group. Selective broadening of peak e allows us to assign it to -COOH of 6-PGA. The transient nature of peaks c and d, as well as the lack of any effect of Mn(II) on the line width these resonances are commensurate with our assignment of these two resonances to >C=O group of 6-PGL(s). In this experiment it was not possible to follow the concentrations of various species as the observed intensities of >C=O carbons were low due to their long spin-lattice relaxation times*. Fig 3 shows an analogous experiment where 2-[^{13}C]glucose was used. α and β anomers of Glc6p (and/or glucose) give resonances at 74.4 and 77.1 ppm (peaks a and b in Fig. 3). Transient resonances at 73.9 and 75.3 ppm (peaks c and d in Fig. 3) are due to lactones and peak e at 76.9 ppm is due to 6-PGA. Since there is no change in the number of hydrogens directly attached at C-2 position of the sugars in this oxidation reaction, spin-lattice relaxation times of ^{13}C-2 of precursors and products remain in the same range. Thus the intensities of various ^{13}C resonances arising from C-2 of various sugars can be approximated to their actual concentrations. This permits us to quantitate the changes in the concentrations of lactones and 6-PGA with time. Fig. 4 compiles such data. Here, the intensities of various resonances have been plotted as a function of time. The steady state nature of the intermediates precludes derivation of true first order rate constants for decay in the intensities of peaks c and d. However, apparent rate constants derived for the decrease in the concentration of c and d resonances are 4.1×10^{-4} and 8.2×10^{-4} s^{-1} respectively. These rate constants are very similar to the ones reported earlier for 6-PGL(s) [4].

* One of the problems in using NMR resonance signal intensity as direct measure of the concentration is the possibility of partial saturation due to rapid pulsing during data acquisition Particularly so in the case of carbonyl groups, which are generally characterized by rather long ^{13}C-T_1 relaxation times, e.g. for an aqueous solution of 6-PGA, T_1 for >C=O carbon is ~10 s which would require extremely long delay periods in order to record this resonance at its full intensity

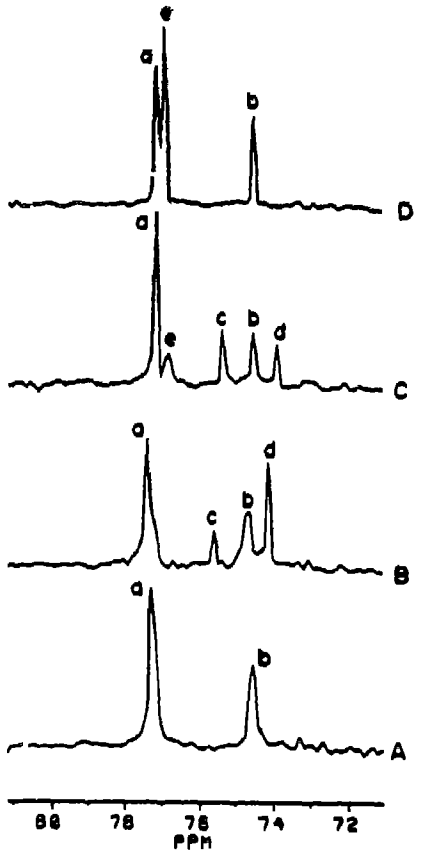

Fig. 3. Characterization of products of G6PD reaction by ^{13}C-NMR using 2-[^{13}C]glucose See section 2 for details Spectrum (A) before addition of G6PD, (B) 3 3, (C) 13.2 and (D) 175 min after the addition of G6PD. Chemical shifts of various resonances are (a) 77 1, (b) 74 4, (c) 75 3, (d) 73.9 and (e) 76 9 ppm.

3.1. *Assignments of resonances*

In the conversion of Glc6p to 6-PGL with G6PD, C-1 of Glc6p which is an asymmetric carbon (and hence Glc6p exists as α and β anomers), loses this asymmetry. In the NMR spectrum (^{13}C,^{31}P or ^{1}H), two anomers

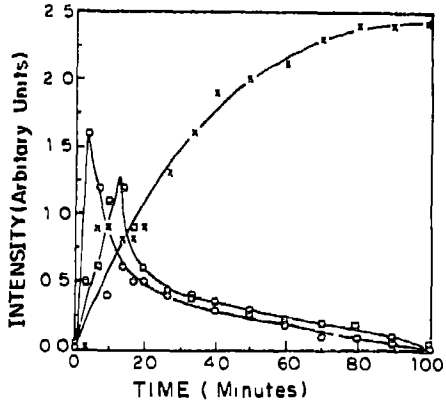

Fig 4 Variation of intensity of peaks c, d and e (from Fig 3) with time Peak c (□—□), peak d (○—○), and peak e (×—×).

give rise to two resonances. However, with the loss of asymmetry at the anomeric carbon, only one resonance should be observed for the lactone. Contrary to this expectation, in all our experiments two resonances which are attributable to lactone(s), are observed (Fig. 1, peaks 5 and 6; Figs. 2 and 3, peaks c and d). The contention that these two resonances arise from lactone(s) is supported by (a) the transient nature of these resonances, (b) addition of bacterial 6-phosphogluconolactonase enhances the rate of disappearance of these resonances and (c) chemical shifts of carbonyl carbon of 1-4 (γ) and 1-5 (δ) gluconolactones are comparable to peaks d and c in Fig. 2 [5]. In an experiment where we studied the rate of spontaneous hydrolysis of δ-gluconolactone (supplied by Sigma), resonance from the >C=O carbon of lactone was observed at 176.9 ppm. These results permit us to assign peak c (176.9 ppm) in Fig. 2 to 1-5(δ) and peak d to 1-4 (γ)-6-phosphogluconolactone.

Intramolecular esterification occurs in hydroxy acids (e.g. 6-PGA) leading to the formation of γ (1-4) and/or δ (1-5) lactones (cyclic esters) with loss of a water molecule. Under acidic conditions, formation of lactone is favoured, whereas treatment with base results in rapid opening up of the ring to give the open chain acid form. In a solution of 6-PGA at neutral pH, we could not detect any lactone by NMR. It has been reported that when 6-PGA is dissolved in 1N HCl, equilibrium is towards the formation of lactones [6]. A sample of 2-[^{13}C]6-PGA was lyophilized and dissolved in 1N HCl. ^{13}C spectrum of this sample showed two resonances at 74.6 and 75.8 ppm. The intensity of resonance at 74.6 ppm was much higher (~3-fold) as compared to the intensity of resonance at 75.8 ppm (data not presented). Since γ-lactones are intrinsically more stable as compared to δ-lactones, higher intensity resonance will be assignable to γ-lactone. On the basis of observed difference in the intensity of two resonances and the apparent rate constants of spontaneous hydrolysis (see above), we can assign resonance c and d (Fig. 3) to γ-lactone and δ-lactones respectively.

3.2. *Mechanism of dehydrogenation*

The dehydrogenation reaction catalysed by G6PD was first thought to proceed via a hydrated adduct of the aldehyde group of Glc6p (2,7) (Scheme I, Fig. 5). This proposal was supported by the demonstration that 6-PGA was the only stable product of the Glc6p oxida-

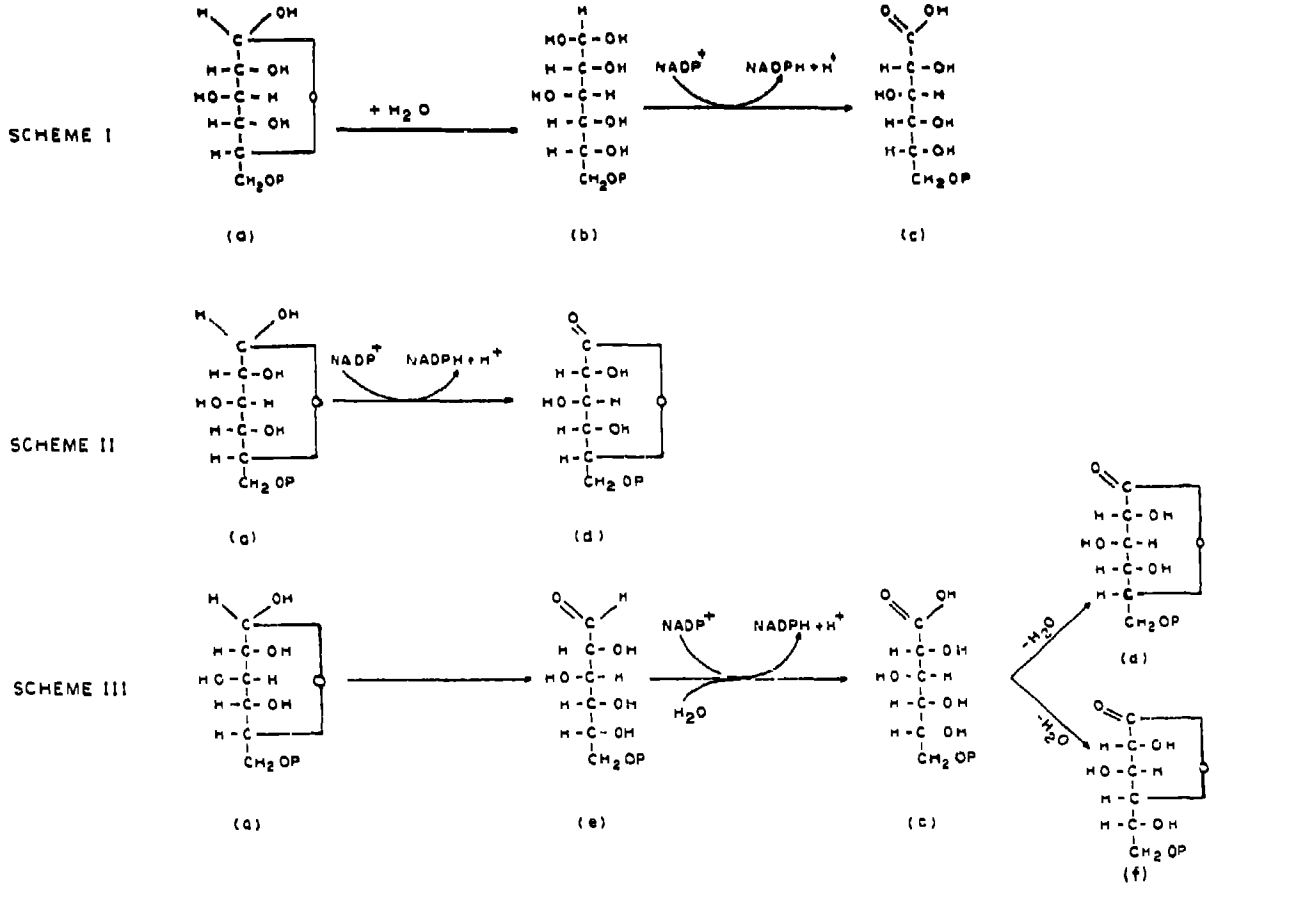

Fig 5. Scheme I and II represent the reaction mechanisms proposed earlier. Scheme III depicts the mechanism consistent with the experimental results presented here (see text for details)

tion. With the realization that Glc6p in solution exists in the pyranose form rather than as the straight chain aldehyde, Cori and Lipmann [3] proposed that the dehydrogenation reaction proceeds directly without the participation of H_2O to yield the unstable δ-lactone as the primary product of the reaction (Scheme II, Fig. 5). However, the unstable lactone was never directly characterized.

We were interested in studying the flux of metabolites through the pentose phosphate pathway in some mutants of yeast using NMR (in vivo). This led us to undertake these experiments to obtain chemical shifts for 1-^{13}C, 2-^{13}C and ^{31}P of 6-phosphogluconolactone. In the course of these experiments, we detected the formation of two lactone products in the G6PD catalyzed oxidation of Glc6p. This observation cannot be explained on the basis of mechanisms proposed earlier (scheme I and II, Fig. 5). Scheme III in Fig. 5 represents a mechanistic proposal to explain the formation of two lactones. The essential feature of this scheme is that although the enzyme may be accepting the pyranose form of Glc6p as a substrate, during catalysis an open chain intermediate (e in Scheme III, Fig 5) must be formed leading to the formation of 6-PGA at the active site of the enzyme. Before release of the product from the active site of the enzyme, intra-molecular esterification occurs. This results in the formation of 1-4 (γ) as well as 1-5 (δ) lactones at the active site which are then released from the enzyme.

Acknowledgements: We thank Drs. G. Krishnamoorthy and Z. Lobo for helpful discussions NMR experiments were carried out at NMR-Facility T.I F.R , Colaba, Bombay 400 005 We thank the facility staff.

REFERENCES

[1] Salas, M., Vinuela, E. and Sols, A. (1965) J. Biol. Chem. 240, 561
[2] Warburg, O., Christian, W and Griese, A. (1935) Biochem. Z., 282, 157
[3] Cori, O. and Lipmann, F. (1952) J. Biol. Chem. 194, 417-425
[4] Bauer, H P., Srihari, T., Jochims, J.C. and Hofer, H.W. (1983) Eur J Biochem. 133, 163-168.
[5] Blazer, R.M. and Whaley, T.W. (1980) J. Am. Chem. Soc. 102, 5082-5085
[6] Brodie, A F. and Lipmann, F. (1955) J. Biol. Chem. 212, 677-685.
[7] Negelein, E and Hass, E (1935) Biochem. Z. 282, 206.