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NON-PROTEIN SUBSTANCES SPECIFICALLY BOUND TO DEHYDROGENASES

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SUMMARY

1. An unidentified substance has been isolated from crystallized α-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12).

2. Ultraviolet and infrared spectra are presented for this substance and the decrease of the maximum at 256 mμ in alkaline solution is typical.

3. It had pK_a values between 9.3 and 9.4, 7.5 and 4.5 and between 0 and 2.

4. Elementary analyses have shown it to contain 5 carbon atoms per nitrogen atom, and it contains no ribose, phosphorus or sulphur.

5. A very similar, if not idential substance was isolated from crystallized α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8).

The electrophoretic and spectral properties of these substances proved them to be quite different from the 3-(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid that VAN EYS found in a α-glycerophosphate dehydrogenase.

INTRODUCTION

More and more researchworkers are reporting the appearance of non-protein substances so tightly bound to animal dehydrogenases, that they can not be removed by ammonium sulphate precipitation, dialysis or ionic-exchange chromatography. They can, however, be separated from the native protein by adsorption on charcoal or by chromatography in urea solution.

WIELAND *et al.*[1] have reported that lactate dehydrogenases (L-lactate: NAD oxidoreductase, EC 1.1.1.27) from different origins all contain approx. 1 mole of NADH-X per mole of enzyme as the only protein-bound substance. Experiments with malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37), carried out in the same institute, also pointed to the presence of a then unknown non-protein component[2].

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VELICK and coworkers[3,4] contended that rabbit muscle α-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) contained 2 moles of NAD per mole of enzyme (mol. wt. 120 000), while RACKER *et al.*[5] thought it to be 3.4–3.6 moles per mole of enzyme in the case of freshly isolated, once recrystallized enzyme. More recently PFLEIDERER AND STOCK[6] showed that α-glyceraldehyde-3-phosphate dehydrogenase contains 2 moles of NAD end 1 mole of NADH-X per mole of enzyme (mol.wt. 117 000). They stated, however, that the NADH-X was formed from NAD during the process of isolating the enzyme and proved this to be correct by considerably shortening the isolation process and finding exactly 3 moles of NAD per mole of enzyme.

ANKEL *et al.*[7] found that α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8) contained 1 mole of ADP-ribose per mole of enzyme (mol.wt. 70 000). VAN EYS *et al.*[8,9] on the other hand found that the same enzyme contained a "prosthetic group" that could replace the thiazole moiety of thiamine in the growth of *Leuconostoc mesenteroides*. They thought the most probable structure to be 3-(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid, synthesized it and found it to be identical with the natural substance in all chemical respects. NAD with its nicotinamide moiety replaced by 4-methyl-5-(β-hydroxyethyl)-thiazole was found to be a reasonably potent inhibitor for a number of enzymes[10].

The present paper deals with the non-protein components of D-glyceraldehyde-3-phosphate dehydrogenase and L-glycerophosphate dehydrogenase and more specifically with a hitherto undiscovered third band fo the non-protein component of D-glyceraldehyde-3-phosphate dehydrogenase, as well as a similar substance found in the non-protein component of L-glycerophosphate dehydrogenase.

EXPERIMENTAL

The D-glyceraldehyde-3-phosphate dehydrogenase and L-glycerophosphate dehydrogenase enzymes were isolated from a freshly prepared homogenate of rabbit muscle according to the method of BEISENHERZ *et al.*[11]. D-Glyceraldehyde-3-phosphate dehydrogenase was a three-times washed and three-times recrystallized preparation, whereas the L-glycerophosphate dehydrogenase was washed three times and recrystallized once. Enzymic activities were determined as described by BEISENHERZ *et al.*[11] using as substrate a triosephosphate mixture prepared by the method of BAMANN AND MYRBÄCK[12]; the measurements were made at 366 mμ and 25° on an Eppendorf photometer equipped with a recording attachment. The bound nucleotides were separated from the protein by adsorption on to acid-washed Nuchar-C 190 (unground) charcoal according to the method of LE PAGE AND MUELLER[13].

High-voltage electrophoresis was carried out on a "Pherograph Frankfurt" apparatus (Ludwig Hormuth, Wiesloch) using MN 2214 FF paper (Macherey, Nagel and Co., Düren). At pH 7.5 a 0.2 M $(NH_4)_2CO_3$ buffer was used, prepared by bubbling CO_2 through the solution until it reached a pH of 7.5. During the electrophoresis a slow stream of CO_2 was constantly bubbled through the buffer in the cathode through, to keep the pH constant throughout the paper. The pH-2.0 buffer was made by mixing 150 ml of glacial acetic acid (A.R.) and 50 ml of concentrated formic acid (A.R.) with 800 ml of quartz-double-distilled water. Ultraviolet absorbing components

were detected with a short-wave ultraviolet lamp (Mineralight Model SL 2537). A longer wavelength (366 mμ) ultraviolet lamp was also used; since it was observed that only the fastest moving band of each non-protein component absorbed light emitted from this lamp.

Protein was determined by the biuret method of WEICHSELBAUM[14], measuring the extinction at 546 mμ in a 2-cm cuvette and using a factor of 16.5. Ribose was determined by the orcin reaction for pentose[15] and phosphate by the method of HIRATA AND APPLEMAN[16] as well as by the following modification of their method. To a suitable aliquot of the $HClO_4$ digest was added enough 70% (v/v) $HClO_4$ to bring its concentration up to 0.702 N, 0.5 ml 0.4% molybdate (0.5 ml of 0.4% solution) and acetone (2 ml) were added, and the volume was made to 5 ml. To this solution was then added 0.1 ml of a 0.5% solution of $SnCl_2$ and the intensity of the blue colour was measured in a Zeiss spectrophotometer at 740 mμ.

The ultraviolet spectra were measured in a Beckman Model DK 2 recording spectrophotometer and the infrared spectra in a Perkin-Elmar Model 21 spectrophotometer.

RESULTS AND DISCUSSION

Non-protein component of D-*glyceraldehyde-3-phosphate dehydrogenase*

The total freeze-dried nucleotides obtained after the charcoal treatment of 2450 mg of D-glyceraldehyde-3-phosphate dehydrogenase amounted to 132.3 mg. When subjected to electrophoresis at pH 7.5 for 2.5 h at 30 V/cm and 80 mA, the electrophoretogram shown in Fig. 1 was produced. The majority of the ultraviolet absorption was contained in Bands 1, 2 and 3. Band 1 was the only component to absorb, when illuminated with the long-wave ultraviolet lamp, an observation which later served as a criterion for the presence or not of this substance in the nucleotide mixture. As

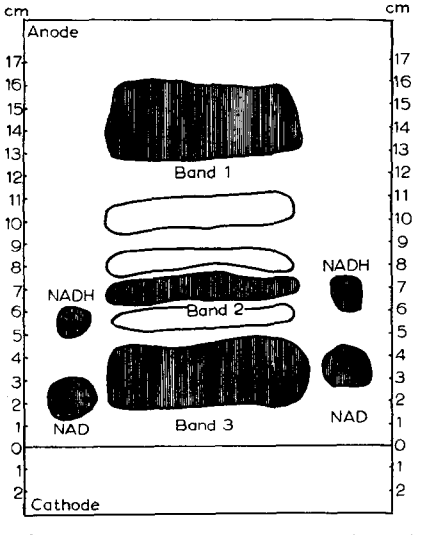


Fig. 1. Electrophoretogram of total D-glyceraldehyde-3-phosphate dehydrogenase nucleotides at pH 7.5.

described in a previous publication Bands 3 and 2 proved to be NAD and NADH-X respectively[6].

After the elution and freeze-drying of Band 1, 108.5 mg of substance was obtained. The ultraviolet spectrum of this substance, as well as that of the total nucleotides, is shown in Fig. 2. With its conspicuous, gradually increasing, absorption from 360 mμ onwards up to its maximum at 243 mμ, it shows a spectrum unlike that of any of the known nucleotides. Chemical analyses established that it contained neither ribose nor phosphate.

To test the homogeneity of the substance, samples were subjected to chromatography in ethanol–0.1 N acetic acid (1:1, v/v) and *n*-propanol–5% $NaHCO_3$ (2:1, v/v).

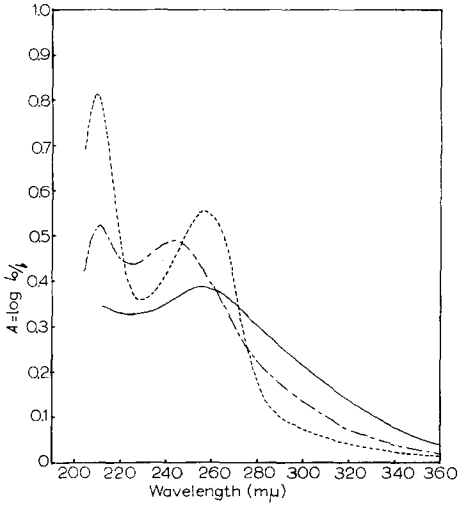


Fig. 2. Ultraviolet spectra of some non-protein components of D-glyceraldehyde-3-phosphate dehydrogenase. - - - - -, total D-glyceraldehyde-3-phosphate dehydrogenase nucleotides; —·—, D-glyceraldehyde-3-phosphate dehydrogenase Band 1, after electrophoresis at pH 7.5; ———, D-glyceraldehyde-3-phosphate dehydrogenase Band 1, after electrophoresis at pH 2.

In both solvents it moved as a uniform spot with R_F values of 0.81 and 0.15 respectively. Since indications were, however, found that more than one spot appeared when *n*-butanol–glacial acetic acid–water (4:1:5, v/v) solvent was used, the main sample was subjected to chromatography on MN 2214 FF paper in this solvent. After running the chromatogram in the cold room for 2 days it was, however, observed that the substance moved with the solvent front and no separation was obtained. Just in front of the main band, and inseparable from it, a second band was observed, which did not absorb under the long-wave ultraviolet lamp. The question now arose whether this second band was present in the substance originally, or whether it was a degradation product formed under the acid conditions to which it was exposed for 2 days.

On account of its strong acid properties in the electrophoretic field and the fact that it contained no phosphorus, the substance was suspected to contain at least two carboxyl groups. It was therefore plausible that under the acid conditions used one or both of these groups were split off to give rise to the second band. If this was so it should

have been possible to separate them electrophoretically. As preliminary experiments have shown that the substance still moved strongly to the anode at pH 2, it was decided to try and separate the two bands electrophoretically. After elution of the chromatogram only 40.5 mg were recovered, which led to the assumption that Band 1 previously still contained some buffer NH_4HCO_3.

Electrophoresis at pH 2 for 2.5 h at 50 V/cm and 45 mA resulted in the electrophoretogram illustrated in Fig. 3. Four nearly equally strong absorbing bands could be detected with the short-wave ultraviolet lamp, while under the long-wave lamp only the fastest moving band, Band 1, could be seen, showing that this was the substance

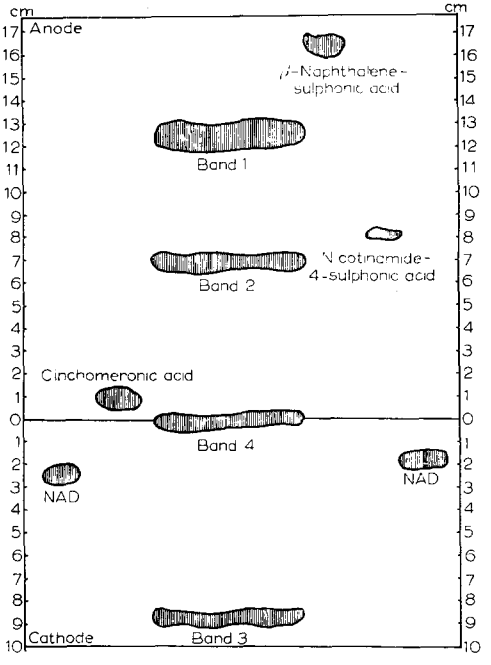


Fig. 3. Electrophoretogram of D-glyceraldehyde-3-phosphate dehydrogenase Band 1 at pH 2.

originally separated at pH 7.5. After elution 11.8 mg were recovered from Band 1 and 5.5, 8.9 and 5.7 mg from Bands 2, 3 and 4 respectively. Since Band 3 moved to the very edge of the paper, it is quite possible that part of it was absorbed into the folio and cloth wick and therefore lost.

The ultraviolet spectra of these bands are reproduced in Fig. 4. Comparing the spectrum of Band 1 with that of the same substance after electrophoresis at pH 7.5, as reproduced in Fig. 2, it can be seen that the maximum shifted from 243 mμ to 256 mμ, and that the absorption between 360 mμ and 256 mμ was much greater. Bands 2, 3 and 4 showed maxima at 256 mμ, 244 mμ, and 258 mμ respectively. At this stage it was noted that the previously discussed second chromatographic band had the same spectrum as Band 3.

The spectral behaviour of Bands 1 and 3 in alkaline and acid solutions is illustrated

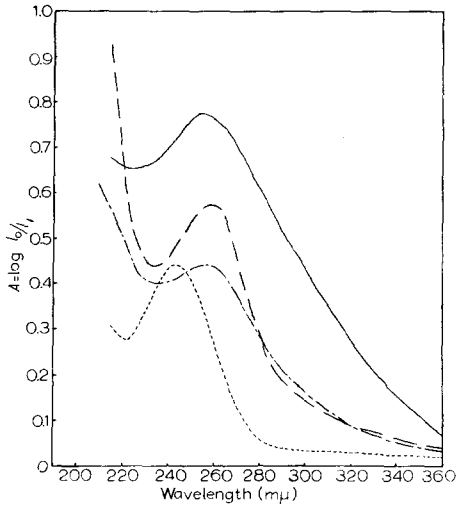


Fig. 4. Ultraviolet spectra of D-glyceraldehyde-3-phosphate dehydrogenase Band 1 components, after electrophoresis at pH 2. ———, Band 1 (*ca.* 0.1 mg per 2 ml of H_2O, pH 5.5); — — —, Band 2 (*ca.* 0.15 mg per 2 ml of H_2O, pH 5.5); - - - - -, Band 3 (*ca.* 0.085 mg per 2 ml of H_2O, pH 6.0); — — —, Band 4 (*ca.* 0.28 mg per 2 ml of H_2O, pH 5.5).

in Figs. 5 and 6 respectively. At pH 1.5 the maximum of Band 1 was slightly decreased. It tended to increase again on standing but remained constant after 5 min. If the pH was raised to 13 the maximum immediately disappeared. Again the absorption tended to increase but also remained constant after 5 min. On adjusting the solution to pH 1.5 the maximum slowly reappeared, the original spectrum being reached after about 1 h with an isosbestic point at 330 mμ.

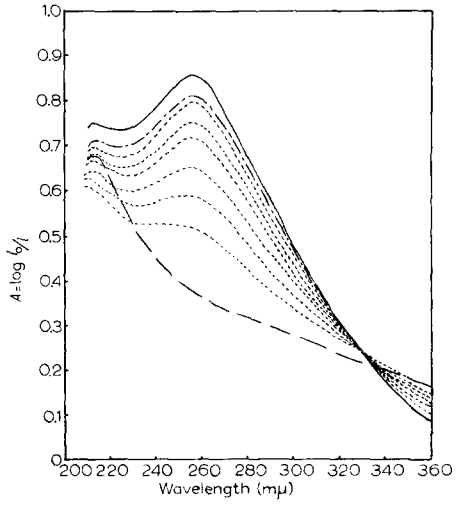


Fig. 5. Spectral changes of D-glyceraldehyde-3-phosphate dehydrogenase Band 1 obtained after electrophoresis at pH 2 (*ca.* 0.1 mg per 2 ml of H_2O). ———, at pH 5.5; — — —, at pH 1.5; — —, at pH 13; - - - - -, spectral changes on standing.

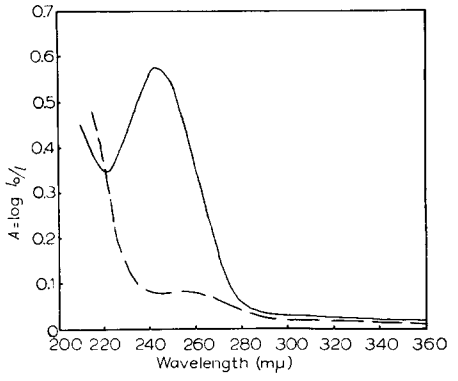


Fig. 6. Spectral changes of D-glyceraldehyde-3-phosphate dehydrogenase Band 3, obtained after electrophoresis at pH 2 (*ca.* 0.13 mg per 2 ml of H_2O). ———, at pH 6; — —, at pH 13.

In contrast to this, the maximum of Band 3, which also disappeared at pH 13, could not be restored again by acidifying the solution.

Spectral studies at different pH values showed Bands 1 and 3 to have pK_a values between 9.3 and 9.4 and between 9.5 and 9.6, respectively. Electrophoretic studies with β-naphthalenesulphonic acid as marker revealed a second pK_a value for Band 1 which was not observed during the spectral studies. Since the distance that β-naphthalenesulphonic acid moves from the origin is independent of the pH of the buffer used, and since Band 1 moved faster than the β-naphthalenesulphonic acid at pH 7.5, but slower at both pH 4.5 and 2, it must have had a second pK_a value somewhere between pH 7.5 and 4.5. That it must have had a third pK_a value between 0 and 2 can be deduced from the fact that at pH 0 it remained at the origin.

Micro elementary analyses* of Bands 1 and 3 showed them to have the composition as illustrated in Table I.

TABLE I

CHEMICAL COMPOSITION OF BANDS 1 AND 3

	Carbon (%)	*Hydrogen (%)*	*Nitrogen (%)*
Band 1	31.1	5.0	7.4
Band 3	35.2	5.6	10.8

Neither sulphur nor phosphorus could be detected. Presuming the remaining percentage to be oxygen the following emperical formulae could be calculated: Band 1: $C_5H_9NO_7$ and Band 3: $C_4H_7NO_4$.

Since the substances were slightly hygroscopic there was some doubt as to the amount of hydrogen and oxygen present. It is, however, acceptable that for each nitrogen atom Band 1 contained 5 carbon atoms and Band 3 only 4. This again indicated that Band 3 might have been formed out of Band 1 after splitting off of a

* Analyses carried out by Dr. W. J. KIRSTEN, Mikroanalyslaboratoriet, Uppsala Universitets, Medicinsk-Kemiska Institution, Uppsala (Sweden).

carboxyl group. If the nitrogen atom were positively charged at pH 2 the movement of Band 3 to the cathode and of Band 1 to the anode would be explained.

Further proof that Bands 1 and 3 must have contained carboxyl groups was supplied by their infrared spectra, which are illustrated in Figs. 7 and 8. The absorption between 5.9 and 6.5 μ should be attributed to carboxylate groups. These groups unfortunately absorbed so strongly that they masked the absorption of any other groups which might have absorbed in the neighbouring region. Attempts to suppress the

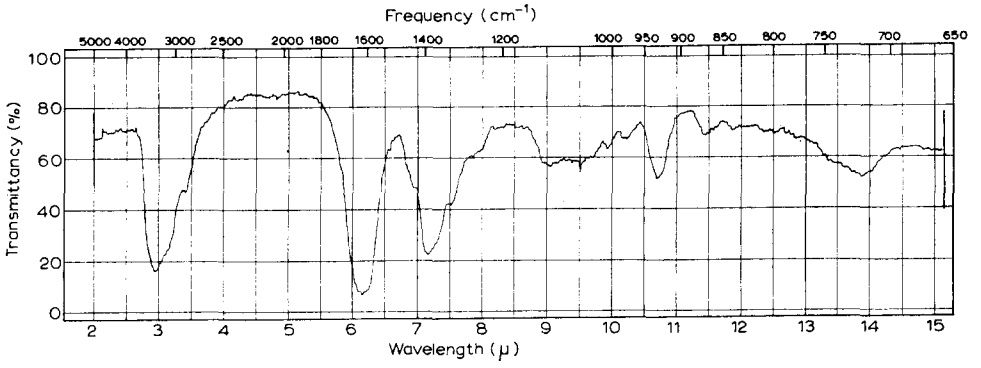


Fig. 7. Infrared spectrum of D-glyceraldehyde-3-phosphate dehydrogenase Band 1, after electrophoresis at pH 2.

absorption by methylating the carboxyl groups with diazomethane, however, met with no success.

In an endeavour to find the possible physiological role of the substance, the inhibitory effect, if any, thereof was investigated. A small sample of the enzyme was treated with charcoal to separate off the nucleotides and a suitably diluted aliquot of the apoenzyme was then used. An eight-fold excess of the substance over the NAD used, could only cause a 30% inhibition. It should be stated, however, that in preparing the apoenzyme it was apparently somewhat degenerated, despite the fact that

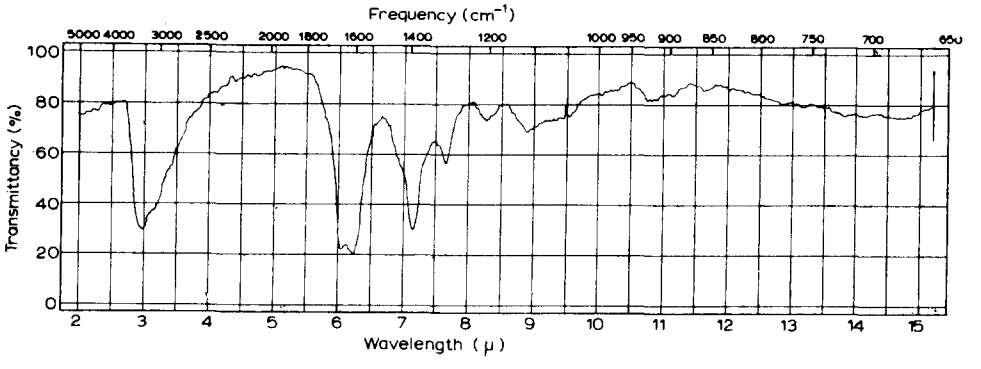


Fig. 8. Infrared spectrum of D-glyceraldehyde-3-phosphate dehydrogenase Band 3, after electrophoresis at pH 2.

gluthathione was added as stabilizing agent, since the apoenzyme had a turnover number of only 1935, whereas the holoenzyme had a turnover number of 3140. That only a small inhibitory effect was observed may therefore possibly be attributed to this partial degeneration.

Non-protein component of L-*glycerophosphate dehydrogenase*

The freeze-dried, charcoal eluate prepared from L-glycerophosphate dehydrogenase was subjected to electrophoresis at pH 7.5 for 2.5 h at 30 V/cm and 80 mA. NAD, NADH, cinchomeronic acid, ADP-ribose, β-naphthalenesulphonic acid, and a sample of purified D-glyceraldehyde-3-phosphate dehydrogenase substance were

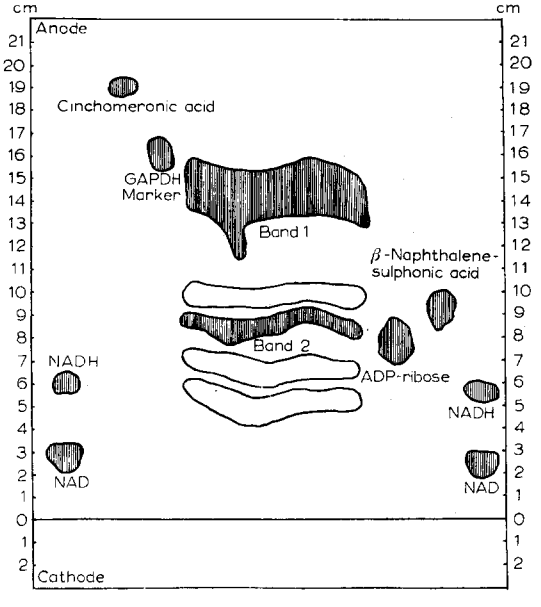


Fig. 9. Electrophoretogram of total L-glycerophosphate dehydrogenase nucleotides at pH 7.5.

used as markers. As can be seen from Fig. 9 the majority of the ultraviolet absorption was contained in Bands 1 and 2. The slower band, Band 2, moved the same distance as the ADP-ribose spot. Analyses for ribose and phosphate, as well as spectral studies, showed this band to be the ADP-ribose found by ANKEL *et al.*[7] The faster band, Band 1, moved as far as the D-glyceraldehyde-3-phosphate dehydrogenase marker. When viewed under the long-wave ultraviolet lamp, only two spots could be detected, the D-glyceraldehyde-3-phosphate dehydrogenase marker and the L-glycerophosphate dehydrogenase Band 1. As was the case with the D-glyceraldehyde-3-phosphate dehydrogenase substance the L-glycerophosphate dehydrogenase Band 1 also contained no ribose or phosphate.

The spectra of the total nucleotides, as well as that of ADP-ribose, are reproduced in Fig. 10. Due to the fact that so little enzyme was available, very little Band-1

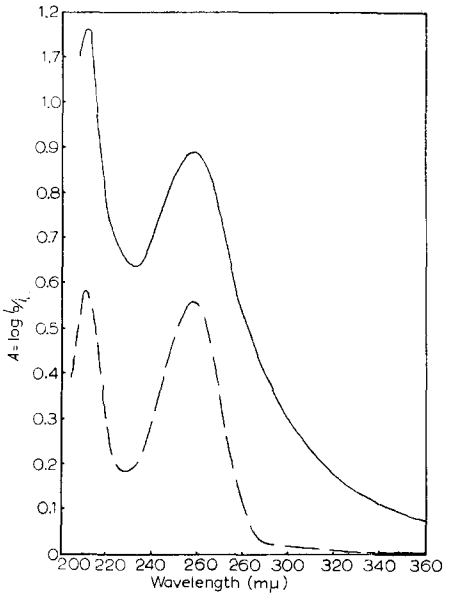


Fig. 10. Ultraviolet spectra of some non-protein components of L-glycerophosphate dehydrogenase. ———, total nucleotides; — —, ADP-ribose.

substance could be recovered, which made it impossible to obtain it pure from the buffer medium. However, from the difference between the two spectra it can still be seen that Band 1 had the same curious increasing absorption in the longer-wavelength region as the D-glyceraldehyde-3-phosphate dehydrogenase substance.

Fig. 11 illustrates the spectral changes of the total nucleotides at different

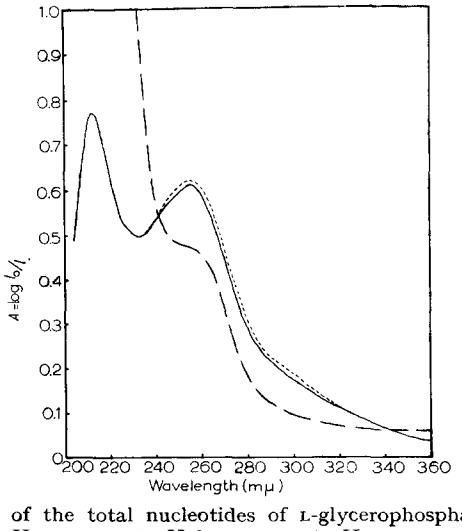


Fig. 11. Spectral changes of the total nucleotides of L-glycerophosphate dehydrogenase with change in pH. ———, at pH 6; - - - - -, at pH 3; — —, at pH 13.

pH values. Since ADP-ribose practically does not change its spectrum with change in pH, the lowering of the maximum at alkaline pH values must be a property of the second component, which again is in accordance with the spectral behavior of the D-glyceraldehyde-3-phosphate dehydrogenase substance.

Electrophoresis at pH 2 for 2.5 h at 50 V/cm and 45 mA resulted in the electrophoretogram, reproduced in Fig. 12. Only three bands were observed, the band that moved to the cathode in the case of D-glyceraldehyde-3-phosphate dehydrogenase not being detected. Assuming the D-glyceraldehyde-3-phosphate dehydrogenase and

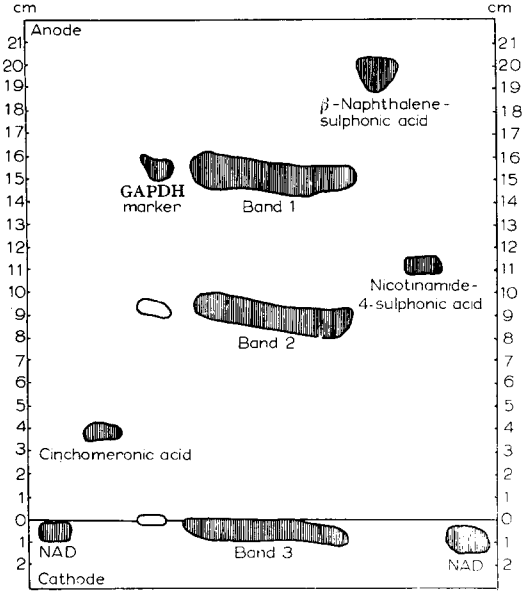


Fig. 12. Electrophoretogram of L-glycerophosphate dehydrogenase Band 1 at pH 2.

L-glycerophosphate dehydrogenase bands to be the same substance, the absence of the fourth band could be ascribed to two possible reasons. It either moved right off the paper or more probably it did not exist at all, since the L-glycerophosphate dehydrogenase substance was not subjected to the acid conditions of chromatography as was the D-glyceraldehyde-3-phosphate dehydrogenase band. The D-glyceraldehyde-3-phosphate dehydrogenase marker also showed three spots only, although the spots coinciding with L-glycerophosphate dehydrogenase Bands 2 and 3 were very weak indeed. That the D-glyceraldehyde-3-phosphate dehydrogenase substance showed more than one spot, must be ascribed to a slow degeneration thereof on storage in solution at pH 5.5 and 4° or under the conditions of electrophoresis. Once again the fastest band, Band 1, and its coinciding D-glyceraldehyde-3-phosphate dehydrogenase marker spot were the only spots visible under the long-wave ultraviolet lamp. These two electrophoretograms also showed that both the D-glyceraldehyde-3-phosphate dehydrogenase and L-glycerophosphate dehydrogenase substances, moved faster than β-naphthalenesulphonic acid at pH 7.5 but slower at pH 2. Although the D-

glyceraldehyde-3-phosphate dehydrogenase Band 1 could not be compared with β-naphthalenesulphonic acid at pH 4.5, due to the fact that too little was available, it presumably also has a pK_a value between pH 7.5 and 4.5.

The whole of Band 1 was then used for measuring the infrared spectrum reproduced in Fig. 13. As can be seen it is similar to that of the D-glyceraldehyde-3-phosphate dehydrogenase substance and also shows the carboxylate absorption maximum between 5.9 and 6.5 μ.

When all the evidence obtained from the electrophoretic, ultraviolet and infrared spectral studies was taken into consideration, it was concluded that the L-glycero-

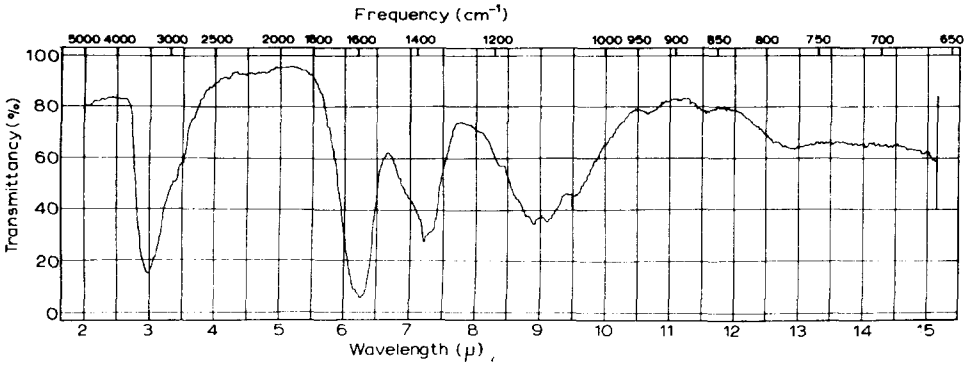
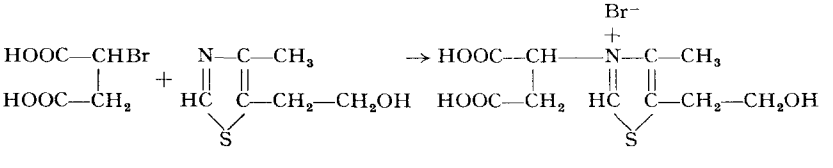


Fig. 13. Infrared spectrum of L-glycerophosphate dehydrogenase Band 1, after electrophoresis at pH 2.

phosphate dehydrogenase substance must be very similar to, if not the same as, the D-glyceraldehyde-3-phosphate dehydrogenase substance.

Electrophoretic and spectral properties of 3-(4-methyl-5-(β-hydroxyethyl)thiazolyl) succinic acid

Since VAN EYS[9] contended 3-(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid to be the prosthetic group of L-glycerophosphate dehydrogenase, the compound was synthesized and its electrophoretic and spectral properties were compared with those of the substances L-glycerophosphate dehydrogenase. A modification of the method of CLINE *et al.*[17] was used, the 4-methyl-5-(β-hydroxyethyl)-thiazole being dissolved in absolute alcohol and an excess of bromosuccinic acid being added. The mixture was boiled in a water-bath under reflux for 1 h, after which the alcohol was evaporated, water was added and the mixture was neutralized with concentrated NaOH.



Electrophoresis of the reaction mixture at pH 7.5 resulted in three distinct bands, one that did not move from the start (Band 3), one that moved a long way to the anode (Band 1), and one in between (Band 2). Because bromosuccinic acid has two negative charges at pH 7.5, the succinic acid thiazolyl compound, two negative and one positive charge, and the thiazole compound no charge, it was expected that Band 1 was bromosuccinic acid, that Band 2 was the thiazolyl succinic acid, and that Band 3 was the unchanged thiazole. That this, indeed, was so was proved by the ultraviolet spectra of the three bands. Bands 1 and 3 had exactly the same spectra as bromosuccinic acid (maximum 213 mμ) and the thiazole compound (maxima 209 and 250 mμ) respective-

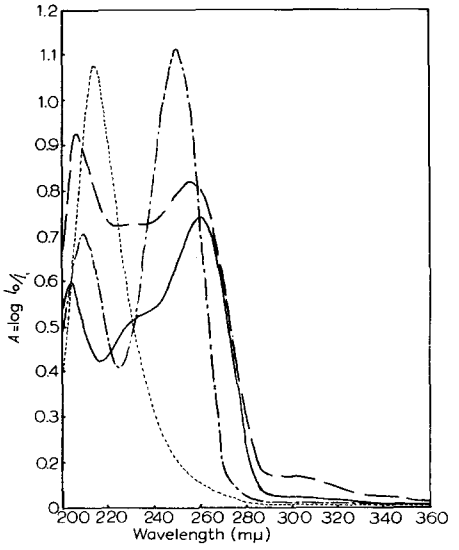


Fig. 14. Ultraviolet spectra of the thiazolyl succinic acid compound and its two components. - - - - -, bromosuccinic acid; — — —, 4-methyl-5-(β-hydroxyethyl)-thiazole; — —, 3-(4-methyl-5(β-hydroxyethyl)-thiazolyl) succinic acid after electrophoresis at pH 7.5; ———, 3(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid after electrophoresis at pH 2.

ly, whereas Band 2 showed a fully different spectrum with maxima at 256 and 206 mμ (Fig. 14).

When Band 2 was eluted and subjected to electrophoresis at pH 2 three bands were observed, a faint band that did not move away from the origin, a distinct band that moved 3 cm to the cathode, and a third, faint band that moved 9 cm to the cathode. The 3-cm band, the thiazolyl succinic acid, had a spectrum with maxima at 204 and 260 mμ as is illustrated in Fig. 14.

Its spectral changes in alkaline, and acid, solution are illustrated in Fig. 15. The increased absorption and the shifting of the maximum from 260 mμ to 234 mμ at pH 13 are typical spectral changes associated with the opening of the thiazolyl ring and are quite contrary to those which are observed with the D-glyceraldehyde-3-phosphate dehydrogenase and L-glycerophosphate dehydrogenase substances.

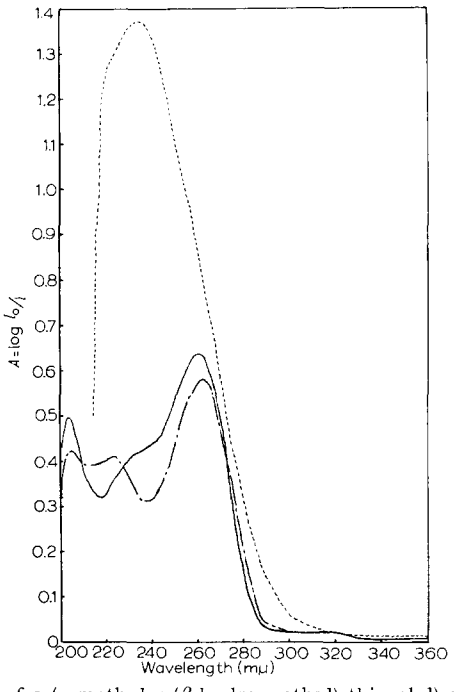


Fig. 15. Spectral changes of 3-(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid with change in pH. ———, at pH 6; —·—, at pH 1.5; - - - - -, at pH 13.

Fig. 16 shows the infrared spectrum of the thiazolyl compound, the carboxylate absorption between 5.9 and 6.5 μ again being evident.

These electrophoretic and spectral studies therefore convincingly prove that the substances found in D-glyceraldehyde-3-phosphate dehydrogenase and L-glycerophosphate dehydrogenase can not possibly be the thiazolyl succinic acid found by VAN EYS in L-glycerophosphate dehydrogenase. All other attempts to identify the substances failed. However, it was noted that ZILLIKEN[18] published a spectrum very

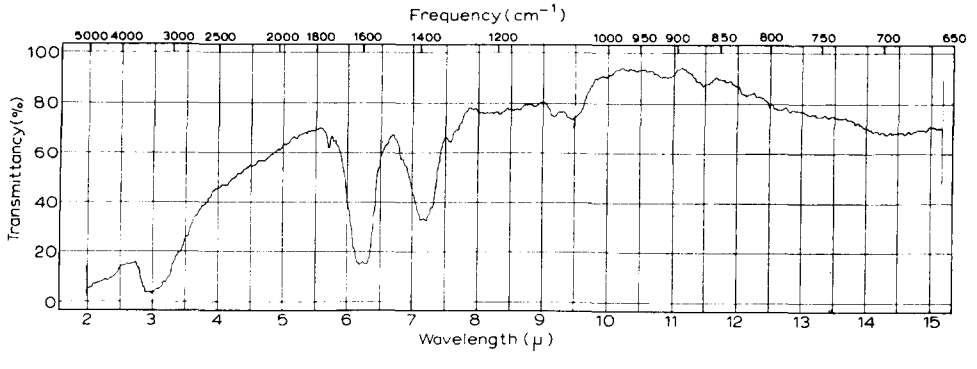


Fig. 16. Infrared spectrum of 3-(4-methyl-5-(β-hydroxyethyl)-thiazolyl) succinic acid, after electrophoresis at pH 2.

similar to that of the D-glyceraldehyde-3-phosphate dehydrogenase substance. His spectrum was obtained after acid hydrolysis of a chondrogenic factor extracted from chicken embryos. He thought that this substance was a derivative of pyrrole-α-carboxylic acid. Spectral studies with a derivative of pyrrole-dicarboxylic acid, however, failed to reveal any similarity with the above-mentioned substances. Further studies are now being undertaken to identify these substances.

REFERENCES

1 TH. WIELAND, P. DUESBERG, G. PFLEIDERER, A. STOCK AND E. SANN, *Arch. Biochem. Biophys., Suppl.* 1 (1962) 260.
2 G. PFLEIDERER, E. HOHNHOLZ-MERZ AND D. GERLACH, *Biochem. Z.*, 336 (1962) 371.
3 S. F. VELICK, J. E. HAYES, JR. AND J. HARTING, *J. Biol. Chem.*, 203 (1953) 527.
4 J. F. TAYLOR, S. F. VELICK, G. T. CORI, C. F. CORI, AND M. W. SLEIN, *J. Biol. Chem.*, 173 (1948) 619.
5 E. RACKER, V. KLYBAS AND M. SCHRAMM, *J. Biol. Chem.*, 234 (1959) 2510.
6 G. PFLEIDERER AND A. STOCK, *Biochem. Z.*, 336 (1962) 56.
7 A. ANKEL, TH. BÜCHER AND A. CZOK, *Biochem. Z.*, 332 (1960) 315.
8 J. VAN EYS, B. J. NUENKE AND M. K. PATTERSON, JR., *J. Biol. Chem.*, 234 (1959) 2308.
9 J. VAN EYS, *Federation Proc.*, 19 (1960) 26.
10 J. VAN EYS, R. KRETSZCHMAR, NAN SEN TSENG AND L. W. CUNNINGHAM, JR., *Biochem. Biophys. Res. Commun.*, 8 (1962) 243.
11 G. BEISENHERZ, H. J. BOLTZE, TH. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT AND G. PFLEIDERER, *Z. Naturforsch.*, 8b (1953) 555.
12 E. BAMANN AND K. MYRBÄCK, *Die Methoden der Fermentforschung*, Vol. 1, Thieme, Leipzig, 1941, p. 304.
13 G. A. LE PAGE AND G. C. MUELLER, *J. Biol. Chem.*, 180 (1949) 975.
14 T. E. WEICHSELBAUM, *Am. J. Clin. Pathol. Techn. Sec.*, 10 (1946) 40.
15 W. MEJBAUM, *Z. Physiol. Chem.*, 258 (1939) 117.
16 A. A. HIRATA AND D. APPLEMAN, *Anal. Chem.*, 31 (1959) 2097.
17 J. K. CLINE, R. R. WILLIAMS AND J. FINKELSTEIN, *J. Am. Chem. Soc.*, 59 (1937) 1052.
18 F. ZILLIKEN, *13. Kolloquium der Gesellschaft für Physiologische Chemie in Mosbach, Induktion und Morphogenese*, Springer-Verlag, Berlin, 1963, p. 164, Fig. 30.