- Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Rothschild (Lord) & Cleland, K. W. (1952). *J. exp. Biol.* **29**, 66.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Thannhauser, S. J., Boncoddio, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **183**, 417.
Thompson, R. H. S. & Webster, G. R. (1957). *Biochem. J.* **67**, 31 p.

- Uziel, M. & Hanahan, D. J. (1957). *J. biol. Chem.* **226**, 789.
Webster, G. R. (1957). *Nature, Lond.*, **180**, 660.
Witter, R. F., Morrison, A. & Shepardson, G. R. (1957). *Biochim. biophys. Acta*, **26**, 121.
Zeller, E. A. (1951). In *The Enzymes*, 1, part 2, p. 987. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.

Biochem. J. (1960) **75**, 258

Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

THE DEOXYRIBOSE POLYNUCLEOTIDE COMPONENT AND THE PHYSICOCHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME

By C. A. APPLEBY* AND R. K. MORTON†

Department of Biochemistry, University of Melbourne, Australia

(Received 2 July 1959)

The absorption spectrum of reduced and oxidized crystalline cytochrome b_2 (Appleby & Morton, 1954, 1959*a*, *b*) shows a pronounced peak at about 265 m μ . It has been found that this absorption band is mainly due to a deoxyribose polynucleotide which is intimately associated with the enzyme as purified by the method of Appleby & Morton (1959*a*). This paper describes the chemical composition of this component and the nature of its association with the crystalline enzyme.

Electrophoresis and sedimentation studies of the crystalline cytochrome b_2 revealed only one protein component. The separation of nucleotide-depleted enzyme from intact enzyme by electrophoresis is described. The results confirm that yeast lactic dehydrogenase is identical with cytochrome b_2 and is a protein with both haem and flavin prosthetic groups (Appleby & Morton, 1954).

This work was reported by us at the meeting of the Australian and New Zealand Association for the Advancement of Science in Melbourne, 1955. It has been described by Appleby (1957), and brief accounts of some aspects have been published (Appleby & Morton, 1954; Morton, 1955, 1958).

MATERIALS

Crystalline yeast lactic dehydrogenase (cytochrome b_2). This was prepared as described by Appleby & Morton (1959*a*). Unless otherwise stated, the enzyme was recrystallized

several times from a solution of the combined first crystals from a number of preparations.

Deoxyribonucleic acid and deoxyribonucleotides. Polymerized calf-thymus deoxyribonucleic acid (DNA) containing 8.2% of P was kindly supplied by Mr G. L. Ada, and chromatographically pure 5'-deoxyribonucleotides were obtained from the California Foundation for Biochemical Research.

Ribonucleic acid and ribonucleotides. Yeast ribonucleic acid (RNA) (British Drug Houses Ltd.) and chromatographically pure 5'-ribonucleotides (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used.

Alkaline phosphomonoesterase. The purified enzyme from calf intestinal mucosa (Morton, 1954) was used.

Deoxyribonuclease and ribonuclease. Crystalline preparations from pancreas were obtained from Worthington Biochemical Corp., U.S.A.

Bovine serum albumin. Crystalline protein (The Armour Laboratories, U.S.A.) was used.

METHODS

Estimation of enzymic activity. The units of activity and the assay procedures are given by Appleby & Morton (1959*a*).

Chemical determinations

Phosphate. For inorganic phosphate, the procedure of Weil-Malherbe & Green (1951) was modified so that one-third of the amounts of all reagents were used in a final volume of 3.1 ml. With these volumes it was found necessary to shake for 1 min. instead of 15 sec. E was read at 730 m μ .

For total phosphate the sample was digested for 10 min. with 0.12 ml. of 10*N*-H₂SO₄ and 0.05 ml. of 72% (v/v) perchloric acid, the digest was diluted to 1.5 ml. with water, hydrolysed for 10 min. at 100° and cooled. The inorganic phosphate was then estimated, acid being omitted from the molybdate reagent.

* Present address: Division of Plant Industry, C.S.I.R.O., Canberra.

† Present address: Department of Agricultural Chemistry, The Waite Agricultural Research Institute, The University of Adelaide.

Protein nitrogen. Approx. 1 mg. of protein was digested for 18 hr. with 0.2 ml. of 50% (v/v) H_2SO_4 containing copper selenite. After cooling, 3 ml. of water and then 3 ml. of Nessler's reagent (King, 1951) were added with rapid mixing. After 15 min. E was read at 480 m μ . Serum albumin (16.07% of N; Tristram, 1953) was used as a standard.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used as previously described (Appleby & Morton, 1959a).

Ribose. The orcinol procedure as described by Hurlbert, Schmitz, Brumm & Potter (1954) was used except that the tubes were heated for 18 min. for colour development. E was read at 660 m μ . With ribose, adenosine and 3'-adenosine monophosphate, the observed ϵ was 24×10^3 , and with 5'-adenosine monophosphate and adenosine triphosphate it was 32.5×10^3 .

Deoxyribose. The method of Ceriotti (1952) was used, except that the chloroform was purified as described by Martin & Morton (1956). E was measured at 492 m μ . With calf-thymus DNA as a standard, E was 12.2×10^3 for a solution containing m-DNA phosphorus. With deoxyribonucleoside-5'-monophosphates, the following ϵ values were obtained: deoxyadenylic acid, 12.8×10^3 ; deoxyguanylic acid, 12.2×10^3 ; deoxycytidylic acid, 1.6×10^3 ; thymidylic acid, 7.4×10^3 .

Purines and pyrimidines. Whatman no. 1 filter papers were washed according to Hanes, Hird & Isherwood (1952), the treatment with calcium acetate being omitted. The general procedures described by Markham & Smith (1949) and by Markham (1955) were followed. The bases were separated by descending chromatography according to Wyatt (1955). The method of detection, elution and estimation of the separated bases has been described by Martin & Morton (1956).

Physicochemical methods

Electrophoresis. Measurements were carried out by J. Pye, Walter and Eliza Hall Institute, Melbourne, with a Tiselius-type moving-boundary electrophoresis apparatus. A cylindrical-lens system was used for detecting the protein and a mechanically driven syringe for compensating boundaries.

Enzyme solutions were dialysed against the buffer for 10–40 hr. and then transferred to the electrophoresis cell (capacity approx. 2 ml.). Electrophoresis was carried out at 2.5°. Photographs were taken with monochromatic light (546 m μ).

Sedimentation studies. The analyses were carried out with a Phywe ultracentrifuge by I. J. O'Donnell and E. F. Woods, Division of Protein Chemistry, Wool Textile Research Laboratories, C.S.I.R.O., Melbourne. The enzyme was dialysed against the buffer for about 14 hr. and sedimentation was carried out at 15° and at 50 000 rev./min. (approx. 182 000 g). Monochromatic light (546 m μ) was used for photography.

pH. Unless otherwise stated, this was measured at about 20° with a glass electrode.

Spectrophotometry. Measurements were made with a Beckman spectrophotometer, model DU, calibrated as previously described (Appleby & Morton, 1959a).

Dialysis. Unless otherwise stated, all dialysis was carried out anaerobically at 0°, air being displaced by oxygen-free nitrogen. The contents of the cellulose tubing, and the diffusate, were frequently mixed.

RESULTS

Physicochemical investigations

Sedimentation studies. Cytochrome b_2 prepared in the absence of ethylenediaminetetra-acetate (EDTA) and recrystallized once was dissolved (4.6 mg./ml.) in a solution of 0.15M-NaCl and 0.4M-sodium lactate at pH 6.8 and at 0°. In the ultracentrifuge a single component was observed which spread very little during sedimentation (Fig. 1). The boundary of the pink cytochrome, which strongly absorbed the green light used for photography, corresponded exactly with the single peak (Fig. 1).

Similar results were obtained with twice-recrystallized cytochrome b_2 prepared from a different strain of baker's yeast and with EDTA used as a protective agent (Appleby & Morton, 1959a) during purification. The sedimentation coefficients ($S_{20, w}$) were 7.77s and 7.70s respectively for the two analyses.

Electrophoresis studies. Analyses were carried out at pH 7.05, 6.65 and 8.8. The concentration of enzyme at pH 7.05 (3 mg./ml.) and 6.65 (2.2 mg./ml.) was lower than desirable owing to the limited solubility of the enzyme at the highest acceptable ionic strength (I 0.25) at these pH values.

Fig. 1. Ultracentrifuge patterns obtained with twice-crystallized cytochrome b_2 (4.6 mg./ml.) in 0.15M-NaCl–0.4M-sodium lactate, pH 6.8. Sedimentation (right to left) at 50 000 rev./min. at 15° for 20 min. (a) and 70 min. (b).

At pH 7.05 a small component appeared within 30 min. of commencement and moved rapidly towards the anode (Fig. 2). The major component moved slowly towards the anode. It remained symmetrical for 205 min. but some asymmetry was evident after 293 min. (Fig. 2). The boundary of the pink cytochrome corresponded exactly with the major component.

Similar results were obtained at pH 6.65, in 0.04 M-sodium pyrophosphate-0.06 M-lactic acid, I 0.25; field strength was 4.07 v/cm. for 325 min.

At pH 8.8 a relatively large component (corresponding to about 6% of the area of the main peak) appeared within 20 min. and moved rapidly towards the anode (Fig. 3). At 195 min. a second peak appeared just ahead of the main pink protein boundary. At 269 min., with the aid of a black-

Fig. 2. Tracings of electrophoresis patterns of crystalline cytochrome b_2 (3 mg./ml.) in 0.1 M-sodium phosphate-0.05 M-sodium lactate (I 0.25, pH 7.05; field strength 3.40 v/cm.; temp. 2.5°). Ascending boundaries at 39 min. (a) and 293 min. (b). The initial boundary position is marked by the arrow.

Fig. 3. Tracings of electrophoresis patterns of crystalline cytochrome b_2 (10.3 mg./ml.) in 0.1 M-sodium lactate-0.015 M-sodium pyrophosphate (I 0.25, pH 8.8; field strength 4.34 v/cm.; temp. 2.5°). Ascending boundaries at 50 min. (a) and at 269 min. (b). The initial boundary position is marked by the arrow.

Fig. 4. Diagram showing the positions (after compensation of boundaries) of the various fractions (F, N, D and B) obtained by electrophoresis at pH 8.8 (see Fig. 3) for 304 min. Composition of the various fractions is represented by: $\square$, deoxyribose polynucleotide; $\blacksquare$, intact enzyme (deoxyribonucleoprotein); $\boxtimes$, nucleotide-depleted enzyme.

Fig. 5. Absorption spectra of fractions (see Fig. 4) obtained by electrophoresis of crystalline cytochrome b_2 at pH 8.8. Where necessary, dilutions were made in 0.5 M-sodium lactate, pH 6.8. Extinction values are given for the undiluted solutions. Some dilution with buffer occurred during removal of fractions from the electrophoresis cell.

paper mask to reduce exposure on the main peak, a photograph clearly showed the double peak on the ascending boundary (Fig. 3) and an asymmetric peak on the descending boundary.

After 304 min., when the resolution of the two peaks had increased further, electrophoresis was stopped. The boundaries were compensated to the positions shown in Fig. 4 and then four fractions (F, N, B and D) were removed from the cell. The absorption spectra of the fractions are shown in Fig. 5.

Fraction F showed a faint-yellow colour and a characteristic flavin fluorescence but, even after oxidation, no flavin absorption bands could be detected near 375 or 450 $m\mu$ (Fig. 5).

Paper electrophoresis of a portion of this fraction was carried out at 400 v for 18 hr. in 0.1 M-sodium phosphate-potassium phosphate buffer, pH 6.8. A large amount of material which absorbed at 254 $m\mu$ remained at the origin; it was not eluted by treatment with 0.1 N-HCl at room temperature, whereas mononucleotides are readily eluted by this treatment. This material was partly eluted by shaking overnight with 0.1 N-HCl at 30°. The eluate showed an absorption spectrum typical of a nucleotide ($\lambda_{\max.}$, 262 $m\mu$; $\lambda_{\min.}$, 230 $m\mu$; $E_{230\text{ }m\mu}/E_{262\text{ }m\mu}$, 0.21).

Fig. 5 and Table 1 show that the absorption spectrum of fraction N was essentially similar to that of the original enzyme, whereas fractions B and D (see Fig. 4) respectively contained a decreasing amount of nucleotide material. These observations showed that fraction F contained a nucleotide which had separated from a portion of the enzyme.

Polynucleotide component of cytochrome b_2

Phosphorus analyses. The total P of the crystalline enzyme, determined by Dr K. W. Zimmerman (Microanalytical Laboratory, University of Melbourne) was 0.56%; there was no inorganic P. Since cytochrome b_2 contains 0.77% of protohaem

and equimolecular amounts of protohaem and ribo-flavin phosphate (Appleby & Morton, 1954, 1959b), there are approx. 15 g.atoms of total P, comprising 14 g.atoms of nucleotide P and 1 g.atom of ribo-flavin P/mole of haem.

Nucleotide analyses. In preliminary studies, denaturation of the enzyme and extraction of flavin with methanol (66%, v/v) at 38° was found to be unsatisfactory, as the supernatant obtained after centrifuging at 25 000 g contained some denatured haemoprotein and material other than flavin which absorbed u.v. light. At 0°, acidification of an enzyme solution with HCl caused maximum turbidity at pH 4.0 but, on further addition of HCl to 0.25 N, a clear-yellow solution was obtained. Subsequently therefore the riboflavin phosphate was extracted and the protein precipitated as follows.

Solutions of cytochrome b_2 were brought to 0.25 N with perchloric acid at 0° to remove ribo-flavin phosphate (Appleby & Morton, 1954, 1959b) and the precipitates were re-extracted with 0.25 N-perchloric acid at 0°. The precipitates so obtained were then extracted with 0.5 N-perchloric acid at 80° for 1 hr. The colourless supernatants had absorption spectra characteristic of nucleotides ($\lambda_{\max.}$, 265 $m\mu$; $\lambda_{\min.}$, 234–240 $m\mu$) and contained no flavin. Assuming ϵ 10.0×10^3 at 265 $m\mu$ for the nucleotide mixture in perchloric acid (Beavan, Holiday & Johnson, 1955), the results of several such studies indicated that the enzyme contains between 9 and 14 nucleotide units/mole of haem.

Extraction of nucleotides with hot HCl instead of perchloric acid was then investigated. Cytochrome b_2 (5.6 mg.) was suspended in 2 ml. of 0.5 N-HCl at 20°. After heating at 80° for 30 min., a flocculent brown precipitate and a pale-yellow supernatant were obtained. The precipitate was collected by centrifuging and washed by resuspending in 0.5 N-HCl at 80°; E at 265 $m\mu$ of the combined supernatants (2.6 ml.) was 5.52. If all of the flavin of the enzyme was degraded to lumichrome ($\epsilon_{265\text{ }m\mu}$ 31×10^3 ; McNutt, 1954) then E due to nucleotide

Table 1. *Properties of the fractions obtained by electrophoresis of crystalline cytochrome b_2 at pH 8.8*

Recrystallized cytochrome b_2 was dialysed anaerobically (see Methods section) for 10 hr. at pH 8.8. Electrophoresis was then carried out (under the conditions given in Fig. 3) for 304 min. The positions of the various fractions after electrophoresis are shown in Fig. 4. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951) (see Methods section). $E_{u.v.}$ and E_{Soret} refer to the maximum extinctions determined at the appropriate wavelengths shown under $\lambda_{\max.}$.

Fraction	Concn. of protein (mg./ml.)	$\lambda_{\max.}$ ($m\mu$)		$\frac{E_{u.v.}}{E_{\text{Soret}}}$	$\frac{E_{u.v.}}{\text{Protein (mg./ml.)}}$	$\frac{E_{\text{Soret}}}{\text{Protein (mg./ml.)}}$
		U.v. band	Soret band			
F	0.19	260	422.5	17.8	52.8	2.96
N	5.0	265–270	422	0.96	2.70	2.82
B	9.9	272	423	0.67	1.94	2.91
D	8.95	272	423	0.54	1.68	3.12
Enzyme*	10.3	266	423	0.93	2.74	2.96

* After dialysis at pH 8.8 and before electrophoresis.

only would be 4.68. Assuming that $\epsilon_{265\text{ m}\mu}$ for degraded nucleotides is 10.0×10^3 (Beavan *et al.* 1955), then these results suggest that the enzyme contains about 17 moles of nucleotide/mole of haem. The extract contained 15.6 g.atoms of P and the residue 1.0 g.atom of P/mole of haem in the original enzyme.

Identification and estimation of nucleotide bases. In a preliminary experiment, riboflavin phosphate was extracted from cytochrome b_2 with 0.25N-perchloric acid at 0°, and nucleotides were extracted from the residue with 0.5N-perchloric acid at 80° (see above). The extract so obtained was brought to 72% (v/v) with perchloric acid and digested at 100° for 30 min., cooled and adjusted to pH 4.5 with KOH. The potassium perchlorate so obtained was removed by centrifuging. The bases in the digest were separated by paper chromatography (see the Methods section) and identified by comparison with those in similar digests of RNA and DNA applied to the same paper. Adenine, guanine, cytosine and thymine were detected but interference by compounds absorbing non-specifically prevented identification of the pyrimidines with certainty.

The nucleotides were then extracted from a solution of oxidized cytochrome b_2 (containing 21.6 μg . of organic P) with 0.5N-HCl at 80°. A portion (1.84 ml.) of the clear-yellow supernatant (2.04 ml.) was freeze-dried and dissolved in 0.6 ml. of formic acid (88%, v/v) and digested at 175° for 1 hr. (Wyatt, 1955) in ampoules sealed under formic acid vapour and containing equal gas and liquid volumes. After drying over NaOH, the hydrolysate was dis-

solved in 39 μl . of N-HCl. Samples of DNA (2 mg.) and of RNA (2 mg.) were each hydrolysed in 1 ml. of formic acid under the same conditions. For chromatography, 12.5 μl . portions of the enzyme hydrolysate and 6.4 μl . portions of the other hydrolysates were applied to separate strips (about 2 cm. wide) from the one sheet of paper, and the chromatogram was developed with the propan-2-ol-HCl solvent of Wyatt (1955).

Comparison of the u.v.-absorbing spots in the hydrolysates of the enzyme with those of hydrolysates of RNA and DNA, and with published R_f values of purines and pyrimidines (Wyatt, 1955), shows that the enzyme contains only guanine, adenine, cytosine and thymine. Uracil was not detected.

When irradiated at about 360 m μ the chromatogram showed a faint-blue fluorescence close to the expected position of uracil (R_f 0.63) and a faint-yellow-green fluorescence overlying the position of cytosine. These fluorescent spots were probably degradation products of riboflavin phosphate.

With similar eluates from the appropriate portions of the blank strip as references, the absorption spectra of eluates of each of the u.v.-absorbing spots in 0.1N-HCl were determined. The results (Table 2) confirm that the enzyme contains adenine, guanine, cytosine and thymine only. No u.v.-absorbing material was detected in the eluate of the area of paper where uracil, if present in the enzyme, would be expected to occur. The agreement of the absorption spectra of the eluted bases with published figures was good for all except cytosine (see Table 2); λ_{max} for this was at 272 m μ .

Table 2. *Estimation of nucleotide bases in crystalline cytochrome b_2*

The bases were separated from formic acid hydrolysates of recrystallized cytochrome b_2 and calf-thymus deoxyribonucleic acid by paper chromatography, and eluted with 0.1N-HCl as described in the text; the final volume was 1.2 ml. In each case measurements were made against an eluate from an appropriate portion of a blank chromatogram. The literature values are from Beavan, Holiday & Johnson (1955) and are given for the bases at pH 1.0 and at mM concn.

Base	Source	Spectrophotometric measurements					Amount (μmole) from chromatogram of	
		λ_{max} (m μ)	E_{max}	$\lambda_{\text{min.}}$ (m μ)	$E_{\text{min.}}$	$\frac{E_{\text{min.}}}{E_{\text{max.}}}$	DNA	Enzyme
Adenine	Literature	262.5	13.15	229	2.25	0.171	—	—
	DNA	262	0.82	229	0.16	0.195	0.075	—
	Enzyme	262	0.76	229.5	0.207	0.272	—	0.069
Guanine	Literature	248.5	11.4	224	3.55	0.31	—	—
	DNA	248.5	0.613	224.5	0.292	0.476	0.065	—
	Enzyme	249.5	0.213	226.5	0.113	0.530	—	0.023
Cytosine	Literature	276	10.0	238.5	1.2	0.120	—	—
	DNA	274	0.518	239.5	0.109	0.210	0.062	—
	Enzyme	272	0.210	239	0.065	0.310	—	0.025
Thymine	Literature	264.5	7.89	233.5	1.9	0.241	—	—
	DNA	265	0.503	233	0.126	0.250	0.077	—
	Enzyme	264.5	0.363	234	0.118	0.326	—	0.055
Total bases (μmole)					—	—	0.279	0.172
Total P (μmole)					—	—	0.303	0.189
Recovery (%)					—	—	92	91

in the enzyme hydrolysate and at $274\text{ m}\mu$ in the DNA hydrolysate, whereas pure cytosine has λ_{max} at $276\text{ m}\mu$ (Beavan *et al.* 1955). The shift in λ_{max} is probably due to traces of the compound which had a faint-yellow fluorescence and which ran close to the position of cytosine; it is not due to the presence of either 5-methylcytosine or of 5-hydroxymethylcytosine, for which, at pH 1.0, the values of λ_{max} are 283.5 and $279\text{ m}\mu$ respectively (Beavan *et al.* 1955). It is nevertheless possible that very small amounts of these compounds may occur in the enzyme.

The amount of each base in the eluate was estimated from the E at λ_{max} and the extinction coefficients of Beavan *et al.* (1955). Assuming that the original extract of the enzyme contained $20.3\text{ }\mu\text{g.}$ of nucleotide P and $1.3\text{ }\mu\text{g.}$ of P due to riboflavin phosphate, the amount of nucleotide P applied to the chromatogram was estimated as $0.189\text{ }\mu\text{mole}$ (i.e. $20.3 \times 1.84 \times 12.5/2.04 \times 39 \times 31$). Thus the calculated recovery of the bases of the enzyme hydrolysate was 91% (Table 2). The results for the base composition of calf-thymus DNA obtained at the same time (Table 2) agree well with those of Chargaff & Lipshitz (1953) for ox-thymus DNA.

The phosphorus and nucleotide analyses indicate that the enzyme contains approx. 15 nucleotide residues/mole of haem. From the relative proportions of each base in the enzyme hydrolysate, the number of residues of each nucleotide/mole of haem have been calculated and are shown in Table 3.

Identification and estimation of the nucleotide sugar. The solution obtained by extraction of nucleotides from cytochrome b_2 with $0.5\text{ N-perchloric acid}$ at 80° (described above) was used. With the orcinol method of Hurlbert *et al.* (1954), a brown solution was obtained, whereas lime-green solutions were obtained with yeast RNA and with ribose nucleotides similarly treated at the same

time. The absorption spectra of the brown and of the green solutions had no similarity to one another.

With the Ceriotti (1952) method for deoxyribose, the nucleotide extract gave a yellow solution, the absorption spectrum of which was identical with that of a solution obtained from DNA treated similarly. From E at λ_{max} ($492\text{ m}\mu$) and with DNA as a standard, it was calculated that the original enzyme solution, which contained $0.065\text{ mm-enzyme-bound haem}$, also contained $0.766\text{ mm-deoxyribose}$.

The sugar was also identified by paper chromatography. A solution of twice-crystallized cytochrome b_2 was dialysed for further crystallization as described by Appleby & Morton (1959*a*). After collection of the crystals, the remaining mother liquor (which contained some uncrystallized enzyme) was brought to $0.25\text{ N-perchloric acid}$ at 0° to remove flavin. The nucleotides were then extracted from the protein precipitate with $0.5\text{ N-perchloric acid}$ at 80° (described above). The extract, containing about $350\text{ }\mu\text{g.}$ of nucleotide, was freeze-dried. The product was dissolved in 0.1 ml. of diethylbarbiturate-HCl buffer, pH 6.9, containing 0.01 M-MgSO_4 . Small portions of pancreatic ribonuclease and deoxyribonuclease and of alkaline phosphatase were added. Samples of DNA and of RNA were similarly treated. The mixtures were incubated for about 14 hr. at 18° and the resultant mononucleotides were hydrolysed (and proteins were precipitated) as described by Chargaff, Vischer, Doniger, Green & Misani (1949) to obtain the free sugars. Paper chromatography of the sugars was carried out according to Partridge (1948) and development was with butanol-ethanol-water (45:5:49). Papers were sprayed first with periodate and then with p -nitroaniline (Edward & Waldron, 1952). This test is specific for deoxypentose. A yellow spot (R_f 0.35) appeared in the DNA hydrolysate and in the enzyme hydrolysate, but not in the RNA hydrolysate. Thus the nucleotide associated with cytochrome b_2 contains a sugar apparently identical with 2-deoxyribose, the characteristic sugar of thymus DNA (Levene & Mori, 1929).

Table 3. Base composition of the deoxyribose polynucleotide of crystalline cytochrome b_2

The analyses were carried out on recrystallized cytochrome b_2 as described in the text and for Table 2.

Base	Moles/mole of haem		Moles/ 100 g.atoms of P
	Found	Probable no.	
Adenine	5.8	6	40.0
Guanine	1.9	2	13.2
Cytosine	2.1	2	14.6
Thymine	4.6	5	32.0
Total purine (moles)			: 1.14
Total pyrimidine (moles)			
Adenine + thymine (moles)			: 2.60
Guanine + cytosine (moles)			

Separation of cytochrome b_2 from ribose polynucleotides during crystallization

The finding of thymine and deoxyribose in the nucleotide associated with cytochrome b_2 and complete absence of uracil and ribose was not expected. Therefore quantitative sugar analyses were carried out on the mother liquors obtained after first crystallization and subsequent recrystallization of a large batch of enzyme. The results are shown in Table 4.

The mother liquor after first crystallization contained a ribose polynucleotide (presumably RNA),

Table 4. Nucleotide sugars in the mother liquors remaining after crystallization of cytochrome b_2

Cytochrome b_2 was prepared according to Appleby & Morton (1959a). The crystals were dissolved in 0.5M-sodium lactate-0.1 mM-EDTA and recrystallized. Ribose and deoxyribose were estimated (see Methods section) in the mother liquor at each successive crystallization. At stage 1, before crystallization the solution contained cytochrome b_2 equivalent to 0.13 mM-deoxyribose.

Stage	Vol. (ml.)	Concn. of sugar (mM)	
		Ribose	Deoxyribose
1	400	32.4	1.6
2	100	0.007	0.082
3	95	0.002	0.065
4	10	0	0.088

equivalent to 32.4 mM-sugar, but the deoxyribose polynucleotide was only equivalent to 1.6 mM-sugar. The cytochrome b_2 crystallized from this mother liquor contained polynucleotide equivalent to 0.13 mM-deoxyribose in the mother liquor. Hence there is a specific association of the deoxyribonucleotide with crystalline cytochrome b_2 . After recrystallization no ribonucleotide could be detected in the crystalline cytochrome b_2 .

Dissociation of the deoxyribose polynucleotide component from the enzyme

Separation by electrophoresis. The separation of the polymerized deoxyribonucleotide in fraction F by electrophoresis at pH 8.8 has been described. As shown in Table 1, for fraction F the ratio of $E_{u.v.}/\text{protein concn. (mg./ml.)}$ was 52.8 compared with 2.74 in the crystalline cytochrome b_2 .

Fractionation with ammonium sulphate. After dialysis of crystallized cytochrome b_2 against 0.63-saturated $(\text{NH}_4)_2\text{SO}_4$ containing 0.5M-sodium lactate and 0.1 mM-EDTA at pH 6.8, a heavy amorphous pink precipitate appeared, whereas a crystalline precipitate was obtained by dialysis against 0.05M-sodium lactate-0.1 mM-EDTA, pH 6.8. The ratios of $E_{264 \text{ m}\mu}/E_{423 \text{ m}\mu}$ for the crystalline and amorphous precipitates were 0.96 and 0.67 respectively. The supernatant from the amorphous precipitate was enriched in nucleotide, the ratio $E_{262 \text{ m}\mu}/E_{423 \text{ m}\mu}$ being 1.97.

In another experiment, the amorphous precipitate obtained by dialysis of cytochrome b_2 against 0.63-saturated $(\text{NH}_4)_2\text{SO}_4$ containing 0.14M-sodium lactate and 0.07 mM-EDTA at pH 6.0, when dissolved in 0.5M-sodium lactate-0.1 mM-EDTA, pH 6.8, had 93% of the original lactic-dehydrogenase activity of the solution. The ratio $E_{268 \text{ m}\mu}/E_{423 \text{ m}\mu}$ was 0.54, compared with 1.06 for the crystalline enzyme. The supernatant from the amorphous precipitate contained 5% of the original enzymic activity; the ratio $E_{261 \text{ m}\mu}/E_{423 \text{ m}\mu}$ was 7.60, and the

Table 5. Effects of deoxyribose polynucleotide component on the properties of crystalline cytochrome b_2

At stage 1, two separate sacs of cytochrome b_2 (0.5 ml.) were dialysed against 0.7-saturated $(\text{NH}_4)_2\text{SO}_4$ containing 0.1M-sodium lactate and 0.02 mM-EDTA, pH 5.9, at 0° to separate the polynucleotide. At stage 2, the contents of one (unopened) dialysis sac were mixed and then dialysed as for enzyme crystallization. The precipitated nucleotide-depleted enzyme was collected from the contents of the other dialysis sac by centrifuging, dissolved in 0.5M-sodium lactate-0.1 mM-EDTA, pH 6.8, and then also dialysed as for enzyme crystallization. Lactic-dehydrogenase activities are expressed as ferricyanide units determined at pH 8.8 and at 20° with sodium pyrophosphate-HCl-EDTA buffer (see Appleby & Morton, 1959a). Extinction values were measured at the appropriate λ_{max} as shown. Protein was estimated according to Lowry *et al.* (1961) (see Methods section).

Stage	Fraction enzyme	Enzymic activity			U.v. band		Soret band		$\frac{E_{u.v.}}{\text{Protein concn. (mg./ml.)}}$
		Units/mg. of protein	Total units	Recovery (%)†	$\lambda_{\text{max.}}$ (m μ)	E	$\lambda_{\text{max.}}$ (m μ)	$\frac{E_{u.v.}}{E_{\text{Soret}}}$	
1	Original enzyme	5300	10 200	—	264	12.0	423.5	11.25	3.11
	Ppt.	4800	7 500	73	268	4.46	423	7.50	1.72
	Supernatant	—	—	0	260	3.0	422	0.15	—
2	Recombined material*	4620	3 130	31	264	0.182	423	0.170	2.68
	Nucleotide-depleted material†	3025	955	9.5	261	0.230	423	0.152	3.65
		1465	285	3.3	263	0.101	422	0.102	2.6
		1490	940	11.0	268	0.104	422	0.157	1.65

* Corresponds to 0.5 ml. of the original enzyme solution.

† Corresponds to 0.42 ml. of the original enzyme solution.

‡ These values refer to the original enzyme solution.

ratio $E_{261\text{ m}\mu}/\text{protein concn. (mg./ml.)}$ was 51. When the nucleotide-depleted enzyme so obtained was dialysed anaerobically at 0° against 0.05 M-sodium lactate-0.01 mM-EDTA, pH 6.8, for 5 hr., no crystals were obtained. A small amount of amorphous pink precipitate collected by centrifuging at 25 000 g for 20 min. at $0-4^\circ$ contained only 18 % of the enzymic activity of the nucleotide-depleted enzyme; most of the activity was in the supernatant fraction.

Recombination of polynucleotide and flavohaemoprotein. Two separate portions, each of 0.5 ml., of once-crystallized cytochrome b_2 (3.8 mg./ml.) were dialysed against 0.7-saturated $(\text{NH}_4)_2\text{SO}_4$ containing 0.1 M-sodium lactate and 0.02 mM-EDTA at pH 5.9 and at 0° . After 4 hr., amorphous precipitates appeared in each dialysis sac.

The contents of one dialysis sac were centrifuged at 25 000 g for 25 min. at $0-4^\circ$, and the precipitate so obtained was dissolved in 0.6 ml. of 0.5 M-sodium lactate-0.1 mM-EDTA, pH 6.8, and at 0° . This solution of nucleotide-depleted enzyme, and the material in the unopened dialysis sac, were both dialysed against 0.05 M-sodium lactate-0.01 mM-EDTA, pH 6.8.

The turbidity of the contents of the unopened dialysis sac rapidly disappeared as $(\text{NH}_4)_2\text{SO}_4$ was removed. After about 2 hr., however, turbidity again appeared in this unopened sac but not in the other. Dialysis was continued overnight and then both sacs were opened.

The preparation in the unopened sac contained a heavy amorphous precipitate. However, a few crystals, having the appearance characteristic of recrystallized enzyme, were detected. Only slight turbidity appeared in the nucleotide-depleted preparation.

The results of the spectrophotometric and enzymic measurements of the various fractions are shown in Table 5.

Enzymic hydrolysis of the deoxyribose polynucleotide. Crystalline ribo- and deoxyribo-nucleases were dissolved in 0.5 M-sodium lactate-0.05 M- MgCl_2 ,

pH 7.0, to give a concentration of 1 mg./ml. Approx. 0.05 ml. of ribonuclease was added to 0.5 ml. of a dilute solution of cytochrome b_2 in 0.5 M-sodium lactate-0.05 M- MgCl_2 , pH 7.0, and 0.05 ml. of deoxyribonuclease was added to a second portion of the same cytochrome b_2 solution. The control preparation contained cytochrome b_2 and 0.05 ml. of 0.05 M- MgCl_2 only. The three preparations were dialysed against 0.05 M-sodium lactate-0.01 mM-EDTA, pH 6.8. After 14 hr. crystals appeared both in the control and in the preparation incubated with ribonuclease, but not in the preparation incubated with deoxyribonuclease. Whereas enzymic hydrolysis of the deoxyribopolynucleotide prevented crystallization by the standard procedure, it did not cause any appreciable change in the enzymic activity of the preparation (Table 6).

Studies of the incorporation of labelled phosphorus into the nucleotide component of cytochrome b_2

It appeared possible that some exchange or incorporation of inorganic phosphate into the polynucleotide component might occur during oxidation of lactate. This activity was therefore determined by measuring the amount of isotope (^{32}P) incorporated into cytochrome b_2 at pH 7.4 during oxidation of lactate in the presence of mM-sodium phosphate containing ^{32}P , as compared with similar tubes with lactate omitted. The cytochrome b_2 was precipitated at 0° with 50 % (v/v) of acetone, washed with 50 % (v/v) acetone at -15° and then dissolved in 0.01 N- NaOH for determination of radioactivity. Separate experiments were carried out with cytochrome b_2 itself, oxygen, cytochrome c , ferricyanide and methylene blue as electron acceptors. In further experiments, incorporation of ^{32}P into added adenosine diphosphate (to form adenosine triphosphate) was measured, essentially as described by Nielsen & Lehninger (1955). Methylene blue and cytochrome c were used as electron acceptors, with and without a dialysed extract of yeast added as a source of possible transferring

Table 6. *Effect of ribonuclease and deoxyribonuclease on the properties of crystalline cytochrome b_2*

Portions of cytochrome b_2 were incubated with crystalline pancreatic ribonuclease or deoxyribonuclease respectively as described in the text and were then dialysed anaerobically for 14 hr. against 0.05 M-sodium lactate-0.01 mM-EDTA, pH 6.8. Lactic dehydrogenase activities were determined at 20° and at pH 7.4 with 0.05 mM-heart-muscle cytochrome c or at pH 8.8 with ferricyanide as in Table 5 and are expressed in the units of Appleby & Morton (1959a).

Treatment	Enzymic activity						Ferricyanide units* Cytochrome <i>c</i> units
	Total ferricyanide units			Total cytochrome <i>c</i> units			
	Ppt.	Supernatant	Total	Ppt.	Supernatant	Total	
Cytochrome <i>b</i> ₂ only	2835	1500	4335	2340	1240	3580	1.21
With ribonuclease	2230	1464	3694	—	—	—	—
With deoxyribonuclease	0	3600	3600	0	2620	2620	1.37

* This ratio is given for the supernatant material.

enzymes and essential cofactors. However, in no case was there significant incorporation of ^{32}P , either into cytochrome b_2 or into adenosine diphosphate.

Nitrogen and sulphur content of cytochrome b_2

Nitrogen. With five replicates of approx. 1 mg. each, the total N of crystalline cytochrome b_2 was found to be $15.1 \pm 0.4\%$. It was estimated that the dried crystals contained 0.71% of sodium lactate (see Appleby & Morton, 1959b). Hence the lactate-free enzyme contains 15.2% of N. The minimum weight/mole is 80 000 g. (Appleby & Morton, 1959b), so that there is 0.065% of haem N, 0.069% of riboflavin N and approx. 0.9% of nucleotide N (assuming 15 nucleotide residues/mole of enzyme-bound haem). Thus cytochrome b_2 contains approx. 14.2% of amino acid N.

Sulphur. Dr K. W. Zimmerman (Microanalytical Laboratory, University of Melbourne) found that the crystalline enzyme contained 0.74% of S.

DISCUSSION

Homogeneity of crystalline cytochrome b_2

The sedimentation and electrophoresis of the recrystallized enzyme was carried out to determine whether more than one protein was present.

Only one component was detected with the ultracentrifuge (Fig. 1) and, since there was little or no spreading of the boundary, it is unlikely that any protein other than cytochrome b_2 is present. Whereas nucleotide-free and intact cytochrome b_2 were separated by electrophoresis at pH 8.8 (as described above), no such separation was observed during sedimentation at pH 6.8.

The results obtained by electrophoresis are complicated by the dissociation of the polynucleotide component of the enzyme. As shown by Figs. 2 and 3, at pH 7.05 and pH 8.8 some free polynucleotide appeared as a fast-moving anion. At pH 6.65, 7.05 and 8.8 the haemoprotein peak remained symmetrical for a considerable period; the nucleotide-depleted protein fraction was separated only after electrophoresis for 269 min. at pH 8.8 (Fig. 3). The results in Fig. 5 and Table 1 establish that the leading anionic protein fraction, fraction N ($\lambda_{\text{max.}}$, 265 m μ ; $E_{265 \text{ m}\mu}/E_{423 \text{ m}\mu}$, 0.96), is closely similar to the original enzyme ($\lambda_{\text{max.}}$, 266 m μ ; $E_{266 \text{ m}\mu}/E_{423 \text{ m}\mu}$, 0.93) and thus contained intact cytochrome b_2 . However, fractions B and D ($\lambda_{\text{max.}}$, 272 m μ ; $E_{272 \text{ m}\mu}/E_{423 \text{ m}\mu}$, 0.67 and 0.54 respectively) clearly contained nucleotide-depleted enzyme. Since the nucleotide-depleted enzyme, having a lower net negative charge/mole, would migrate more slowly than the intact enzyme, this finding accounts for the asymmetry and the separation of the protein peak after prolonged electrophoresis (Figs. 2, 3).

Since the ratio of $E_{\text{Soret band}}/\text{protein concn. (mg./ml.)}$ was almost constant for all fractions obtained at pH 8.8 (Table 1), the only protein material present was a haemoprotein with the absorption spectrum of cytochrome b_2 . No flavoprotein or any other protein not containing protohaem was detected. The small amount of haemoprotein in fraction F (Table 1, and Figs. 4, 5) undoubtedly arose as a contamination during removal of fraction F from the electrophoresis cell. A trace of riboflavin phosphate, dissociated from the enzyme, was detected in fraction F by fluorescence and by paper chromatography, but no flavoprotein was present (Table 1).

Since the results revealed interaction between the enzyme and the buffer systems, which were of differing composition, the electrophoresis mobilities at different pH values could not be used to obtain the isoelectric point of the protein. The minimum solubility of cytochrome b_2 is at about pH 4.0.

The minimum weight/mole of crystalline cytochrome b_2 as estimated from iron, haem and flavin determinations is approx. 80 000 g. (Appleby & Morton, 1959b). This is a more reliable estimate than the earlier value (97 000 g.) reported by us (Appleby, 1957; see also Dixon & Webb, 1958). The amino acid analyses of the intact enzyme (Appleby, Morton & Simmonds, 1960; Morton, 1958) indicate a minimum weight (with one residue of histidine) of approx. 13 000, which is consistent with a value of 80 000 g./mole of enzyme-bound haem (see Morton, 1958). Nygaard (1958) has recently confirmed our earlier value (97 000 g.) but his preparation appears to be contaminated with ribonucleotides. The value of 230 000 g./mole of haem given by Boeri & Tosi (1956) is undoubtedly too high, probably due to impurities in the enzyme preparation (see Appleby & Morton, 1959b; Boeri & Cutolo, 1958).

From the nucleotide analyses (Table 3) the weight of polynucleotide/mole of enzyme-bound haem is approx. 5000 g. Thus the nucleotide-free enzyme has a minimum weight/mole of approx. 75 000 g. and the polynucleotide comprises about 6% of the dry weight of the crystalline enzyme.

Deoxyribose polynucleotide of crystalline cytochrome b_2

The results presented here conclusively establish that the pronounced absorption of crystalline cytochrome b_2 near 265 m μ (Appleby & Morton, 1954, 1959a, b) is due to a deoxyribonucleotide associated with the crystalline enzyme. In enzyme recrystallized several times, both deoxyribose and thymine were identified by at least two procedures, whereas no ribose or uracil could be detected. Since much ribonucleotide is present before crystallization, but remains in the mother liquor (Table 4),

there is clearly a specific association of the protein of cytochrome b_2 and the deoxyribonucleotide, as reported previously (Morton, 1955, 1958; Appleby, 1957). Nygaard (1958) has since reported the preparation of yeast lactic dehydrogenase containing ribonucleotides. Table 4 shows that ribonucleotides are associated with partially purified preparations of cytochrome b_2 when prepared according to Appleby & Morton (1954, 1959*a*), but remain in the mother liquor on crystallization of the cytochrome b_2 . Pellets of once-crystallized cytochrome b_2 collected from the mother liquor by centrifuging are sometimes overlain by a small amount of white amorphous precipitate composed mainly of ribonucleic acid. This contaminant may also cause an elevated value for the ratio $E_{u.v. \text{ band}}/E_{\text{Soret band}}$ of once-crystallized enzyme (see Appleby & Morton, 1959*b*). However, no ribonucleotide has been detected in recrystallized enzyme and Nygaard's (1958) findings can probably be attributed to non-specific contaminants in his preparation of cytochrome b_2 .

The base composition of the deoxyribonucleotide associated with crystalline cytochrome b_2 is given in Table 3. There are approximately equimolecular amounts of adenine and thymine, and of guanine and cytosine, consistent with the requirement of the theory of Watson & Crick (1953) for DNA. The quotient (moles of adenine + moles of thymine) / (moles of guanine + moles of cytosine) for the deoxyribonucleotide of cytochrome b_2 is 2.60 (from Tables 2 and 3) and that for the DNA of baker's yeast is 1.79 (Zamenhof & Chargaff, 1950). The ratio found for calf-thymus DNA is 1.20 (from Table 2) and is in reasonable agreement with the value of 1.36 reported for ox-thymus DNA by Chargaff & Lipshitz (1953). Hence the deoxyribonucleotide of cytochrome b_2 differs substantially from DNA of yeast and this supports the view that it is a specific component of cytochrome b_2 .

Having regard to the difficulties of choosing appropriate standards there is reasonable agreement of analytical results based on the estimation of nucleotide phosphorus, sugars and of bases. The use of extinction coefficients based on ox-thymus DNA will cause some error in the estimation of nucleotide and of sugar, since the base composition differs substantially from that of the cytochrome b_2 polynucleotide (see above). Moreover, the amount of colour in the Ceriotti (1952) reaction for deoxyribose varies with the nature of the nucleotide. The phosphorus analyses are probably the most reliable and these indicate 14–15 nucleotide units/mole of enzyme haem.

The nucleotide units are undoubtedly polymerized, since the polynucleotide does not pass through a cellulose dialysis sac, and it is not eluted from paper under conditions which elute

mononucleotides (see Results section). Moreover, the polynucleotide migrates as a single anionic component during electrophoresis (Fig. 3), and is depolymerized by deoxyribonuclease (see Results section and Table 6).

The polynucleotide is dissociated by high concentrations of ammonium sulphate (see Results section). Hence cytochrome b_2 prepared by salting-out procedures (Bach, Dixon & Zerfas, 1946; Boeri, Cutolo, Luzzati & Tosi, 1955) will contain little or no deoxyribopolynucleotide.

The slight excess of adenine over thymine (Table 3) may indicate that the crystalline enzyme contains an adenine-containing component, other than the deoxyribopolynucleotide. We have observed a pronounced absorption at about 340 m μ in the clear extract obtained by heating a solution of crystalline cytochrome b_2 in neutral lactate at 100° for 3 min. and dispersing the denatured protein in 0.7-saturated ammonium sulphate at 0–4°. This absorption peak was not detected in extracts obtained by heating with hydrochloric acid or perchloric acid at 80° for 1 hr. It is not due to either lumichrome or pyruvate and it is unlikely to be due to reduced di- (or tri-)phosphopyridine nucleotide, since Boeri & Tosi (1956) found less than 0.1 mole of nicotinic acid moiety/mole of haem in their preparation of yeast lactic dehydrogenase.

Function of the nucleotide

The results (Tables 5 and 6) show that the intact nucleotide is essential for crystallization of cytochrome b_2 as described by Appleby & Morton (1959*a*). After dissociation by salt, the nucleotide apparently does not recombine in the specific manner necessary for crystallization, although an amorphous precipitate is formed. However, as yet no other function has been found for the polynucleotide. It is not essential for dehydrogenase activity.

Cytochrome b of animal tissues appears to be essential for formation of adenosine triphosphate accompanying oxidation of reduced diphosphopyridine nucleotide (see Chance & Williams, 1956). Pinchot (1953, 1957) found that a polynucleotide component is essential for oxidative phosphorylation catalysed by intracellular particles from *Alcaligenes faecalis*. It therefore appeared possible that cytochrome b_2 , having a polynucleotide as an integral part of the enzyme, might catalyse a phosphorylation accompanying lactate oxidation. However, no uptake or exchange of inorganic phosphate could be demonstrated with labelled phosphorus (see Results section).

The relationship between polynucleotides (or nucleic acids) and the synthesis of proteins is now well established [see, for example, Brachet (1957) and Chantrenne (1958)]. The incorporation of

amino acids into the protein of isolated nuclei from calf thymus is dependent on deoxyribonucleic acid (Allfrey, Mirsky & Osawa, 1957). It is possible therefore that the polynucleotide of cytochrome b_2 is a component associated with the synthesis of this haemoprotein.

Nucleotropomyosin, which contains a ribonucleotide, was purified by Hamoir (1951) by precipitation at neutral pH and at low ionic strength, as was cytochrome b_2 . Unlike cytochrome b_2 , however, tropomyosin also crystallizes by similar procedures in the absence of the nucleotide.

Cytochrome b_2 also resembles avidin, a non-crystalline protein which contains about 10% of a deoxyribopolynucleotide (Fraenkel-Conrat, Ward, Snell & Ducay, 1950). Intact avidin is insoluble in water but soluble in 0.2M-potassium phosphate; electrophoresis in potassium phosphate buffer gave a fast-moving anionic nucleotide component and a water-soluble nucleotide-free protein (Fraenkel-Conrat *et al.* 1950; Fraenkel-Conrat, Snell & Ducay, 1952).

SUMMARY

1. Sedimentation of crystalline cytochrome b_2 (lactic dehydrogenase) of baker's yeast in an ultracentrifuge revealed only one component, having a sedimentation coefficient ($S_{20,w}$) of 7.7S.

2. Electrophoresis at pH 6.65, 7.05 and 8.8 showed partial dissociation of a polynucleotide component of the enzyme. Separation of nucleotide-depleted enzyme from intact enzyme was detected after electrophoresis for 269 min. at pH 8.8. There was no separation of a flavoprotein from a haemoprotein. The results indicate that crystalline cytochrome b_2 contains only one major protein component.

3. There are approx. 15 (polymerized) deoxyribonucleotide residues/mole of haem in the crystalline enzyme. The base composition (adenine, 6; thymine, 5; guanine, 2; cytosine, 2 moles) of the polynucleotide differs considerably from that of yeast deoxyribonucleic acid. Recrystallized cytochrome b_2 contains no detectable ribose or uracil, indicating the absence of ribonucleotides.

4. Dissociation of the deoxyribopolynucleotide of cytochrome b_2 by 0.7-saturated ammonium sulphate or by digestion with pancreatic deoxyribonuclease prevented crystallization of the enzyme by anaerobic dialysis at low ionic strength. An amorphous precipitate was formed on recombination of the polynucleotide and nucleotide-depleted enzyme.

5. The nucleotide is not essential for dehydrogenase activity. No uptake or exchange of inorganic phosphate during oxidation of lactate catalysed by the crystalline enzyme could be demonstrated. The possible function of the polynucleotide is discussed.

6. There is approx. 0.56% of phosphorus, 0.74% of sulphur and 15.2% of nitrogen in the crystalline enzyme, and approx. 14.2% of nitrogen in the nucleotide-free enzyme.

7. The properties of cytochrome b_2 , a crystalline deoxyribonucleoprotein, are compared with those of other nucleoproteins.

Part of the financial support for this investigation was given by the Division of Plant Industry, C.S.I.R.O., whose assistance is gratefully acknowledged. We also wish to thank Barrett's Food Company Ltd., Melbourne, for the generous gift of dried yeast. We are grateful to Mr G. Ada and Mr J. Pye, of the Walter and Eliza Hall Research Institute, for carrying out the electrophoresis of the enzyme; and to Mr I. J. O'Donnell and Mr E. F. Woods, of the Division of Protein Chemistry, Wool Textile Research Laboratories, C.S.I.R.O., for carrying out the ultracentrifuging and for estimating the molecular weight of the enzyme.

REFERENCES

- Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957). *J. gen. Physiol.* **40**, 451.
- Appleby, C. A. (1957). Ph.D. Thesis: University of Melbourne.
- Appleby, C. A. & Morton, R. K. (1954). *Nature, Lond.*, **173**, 749.
- Appleby, C. A. & Morton, R. K. (1959a). *Biochem. J.* **71**, 492.
- Appleby, C. A. & Morton, R. K. (1959b). *Biochem. J.* **73**, 539.
- Appleby, C. A., Morton, R. K. & Simmonds, D. H. (1960). *Biochem. J.* **75**, 72.
- Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.
- Beavan, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Boeri, E. & Cutolo, E. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 5-36, p. 60.
- Boeri, E., Cutolo, E., Luzzati, M. & Tosi, L. (1955). *Arch. Biochem. Biophys.* **56**, 487.
- Boeri, E. & Tosi, L. (1956). *Arch. Biochem. Biophys.* **60**, 463.
- Brachet, J. (1957). *Biochemical Cytology*. New York: Academic Press Inc.
- Cerioti, G. (1952). *J. biol. Chem.* **198**, 297.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Chantrenne, H. (1958). *Annu. Rev. Biochem.* **27**, 35.
- Chargaff, E. & Lipshitz, R. (1953). *J. Amer. chem. Soc.* **75**, 3658.
- Chargaff, E., Vischer, E., Doniger, R., Green, C. & Misani, F. (1949). *J. biol. Chem.* **177**, 405.
- Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 479. London: Longmans, Green and Co. Ltd.
- Edward, J. T. & Waldron, D. M. (1952). *J. chem. Soc.* p. 3631.
- Fraenkel-Conrat, H., Snell, N. S. & Ducay, E. D. (1952). *Arch. Biochem. Biophys.* **39**, 80.
- Fraenkel-Conrat, H., Ward, W. H., Snell, N. S. & Ducay, E. D. (1950). *J. Amer. chem. Soc.* **72**, 3826.

- Hamoir, G. (1951). *Biochem. J.* **43**, 146.
- Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
- Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
- King, E. J. (1951). *Quantitative Clinical Biochemistry*, 2nd ed. London: J. and A. Churchill Ltd.
- Levene, P. A. & Mori, T. (1929). *J. biol. Chem.* **83**, 803.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McNutt, W. S. (1954). *J. biol. Chem.* **210**, 511.
- Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 246. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Martin, E. M. & Morton, R. K. (1956). *Biochem. J.* **64**, 221.
- Morton, R. K. (1954). *Biochem. J.* **57**, 595.
- Morton, R. K. (1955). *Soc. biol. Chem., India: Silver Jubilee Souvenir*, p. 177.
- Morton, R. K. (1958). *Rev. pure appl. Chem.* **8**, 161.
- Nielsen, S. O. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 555.
- Nygaard, A. P. (1958). *Biochim. biophys. Acta*, **30**, 450.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Pinchot, G. B. (1953). *J. biol. Chem.* **205**, 65.
- Pinchot, G. B. (1957). *Biochim. biophys. Acta*, **23**, 660.
- Tristram, G. R. (1953). In *The Proteins*, vol. 1, part 1, p. 181. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
- Watson, J. D. & Crick, F. H. C. (1953). *Nature, Lond.*, **171**, 737.
- Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.
- Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 243. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Zamenhof, S. & Chargaff, E. (1950). *J. biol. Chem.* **187**, 1.

Biochem. J. (1960) **75**, 269

The Stimulation of Transphosphorylation by Alkali-Metal Ions

By J. M. LOWENSTEIN

Graduate Department of Biochemistry, Brandeis University, Waltham 54, Massachusetts, U.S.A.

(Received 25 August 1959)

A number of enzyme reactions involving the polyphosphate chain of nucleoside triphosphates show an absolute requirement for, or a stimulation by, alkali-metal ions. This requirement is usually met by potassium ions, but sodium, rubidium and ammonium ions have been reported to serve in some cases. Examples include fructokinase (Lardy, Parks & Ben-Gershom, 1957), myosin adenosine triphosphatase (Kielley, Kalckar & Bradley, 1956; Bowen & Kerwin, 1954), pyruvate kinase (Kachmar & Boyer, 1953) and glutathione synthetase (Snock, Yanari & Bloch, 1953). However, the majority of the enzyme-catalysed reactions which involve nucleoside triphosphates have not been tested for an alkali-metal requirement. Unless it is specifically tested for, such a requirement may easily be missed because many reagents, including adenosine triphosphate (ATP), are commonly used in the form of their sodium or potassium salts. It is likely therefore that many more reactions utilizing adenosine triphosphate possess a requirement for alkali-metal ions than are recognized to do so at present.

In a previous paper (Lowenstein, 1958) a non-enzymic transphosphorylation was described which involves the transfer of the terminal phosphate group of adenosine triphosphate to orthophosphate. The products of the reaction are inorganic pyrophosphate and adenosine diphosphate (ADP). The

reaction shows an absolute dependence on bivalent-metal ions (M^{2+}):

Adenosine triphosphate + orthophosphate

Of those tested, Ca^{2+} , Mn^{2+} and Cd^{2+} ions were the most effective.

The work presented here had two objects: the first was to provide a chemical basis for the alkali-metal requirement of enzyme-catalysed transphosphorylations. The second was to study variations in the amount of pyrophosphate formed in the non-enzymic reaction. These were observed to occur with reaction mixtures identical in every respect, except that different bases (such as potassium hydroxide, sodium hydroxide and diethanolamine) were used to neutralize the solutions of adenosine triphosphate employed in the experiments. A preliminary account of this work has been given (Lowenstein, 1959).

EXPERIMENTAL

Materials and reagents

Diethanolamine salt of adenosine triphosphate. The barium salt of adenosine triphosphate (ATP) was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and from the Nutritional Biochemical Corp., Cleveland, Ohio,