Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

ENZYMIC AND CHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME

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(Received 26 February 1959)

Bach, Dixon & Zervas (1946) found that cytochrome b_2 (Bach, Dixon & Keilin, 1942) was an essential part of the dehydrogenase system of baker's yeast which catalysed the reduction of methylene blue by lactate. Appleby & Morton (1954) reported that purified crystalline lactic dehydrogenase of yeast was a chromoprotein having both protohaem and riboflavin phosphate as essential prosthetic groups and was identical with cytochrome b_2 .

The procedure for purification and crystallization of the enzyme has been published (Appleby & Morton, 1959). Some enzymic and chemical properties of the crystalline material are described here. This work was described at the meeting of the Australian and New Zealand Association for the Advancement of Science in Melbourne, 1955, and some preliminary accounts have been published (Appleby & Morton, 1954; Morton, 1955).

MATERIALS

Crystalline yeast lactic dehydrogenase (cytochrome b_2). This was prepared as described by Appleby & Morton (1959). Unless otherwise stated, the material was recrystallized at least once, and sometimes twice, from a solution of the combined first crystals from a number of preparations.

Hydroxy acids. DL- α -Hydroxy-*n*-butyric acid (Bios Laboratories Ltd.), DL- β -hydroxy-*n*-butyric acid (British Drug Houses Ltd.) and α -hydroxy-iso-butyric acid (Eastman Organic Chemicals Ltd.), at approx. molar concentration, were hydrolysed at 100° for 1 hr. and then adjusted to about pH 7.0 with 5N-NaOH and diluted to 0.5M concentration. Glycolic acid (British Drug Houses Ltd.) was treated similarly except that hydrolysis was omitted. DL-Lactic acid was prepared as described previously (Appleby & Morton, 1959).

Pyridine. Analytical-grade reagent (British Drug Houses Ltd.) was redistilled, the first and last portions of the distillate being discarded.

METHODS

Estimation of enzymic activity. The units of activity and the assay procedures have been described by Appleby &

Morton (1959). For estimation of the optimum pH for activity, the buffer system of Michaelis (1931) was used with crude enzyme preparations and 0.1M-sodium pyrophosphate-acetic acid buffer with crystalline enzyme. All reaction mixtures contained 1 ml. of the appropriate buffer in a final volume of 3 ml. The pH values were determined at the conclusion of each experiment with a glass electrode at room temperature.

Molecular extinction coefficient (ϵ). In this paper, values of ϵ given for cytochromes refer to the extinction coefficients for molar concentration of protein-bound haem.

Chemical determinations

Protohaem. This was estimated as the pyridine haemochromogen. Pyridine (0.6 ml.) was added to a suitable amount of material (as dried powder, aqueous suspension or solution) in a Thunberg-type cuvette (Appleby & Morton, 1959). After gentle shaking, about 0.75 ml. of 0.2N-KOH was added and the volume brought to 3 ml. with water. After evacuation, the cuvette was filled with O_2 -free N_2 and about 3 mg. of $Na_2S_2O_4$ was added from the side bulb. The absorption spectrum was determined after 15 min. The haemochromogen so prepared from crystalline protohaemin had ϵ 33.2×10^3 at 556.5 m μ .

Metals. The analyses with a spark spectrograph were carried out by the Defence Standards Laboratory, Commonwealth Department of Supply, Melbourne. The dried crystals were placed in the hollowed bottom carbon electrode of the apparatus.

Iron was estimated according to Drabkin (1941) except that the final volume of the *o*-phenanthroline complex was brought to 5 ml.

Copper was determined by the following modification of the method of Sandell (1944). The samples (containing about 2 μ g. of copper) were heated with 0.1 ml. of 17.5N- H_2SO_4 until acid fumes appeared and were then digested for about 10 min. until colourless. The tubes were cooled, the contents were diluted with water and then neutralized with ammonia with methyl orange as an indicator. After addition of 0.5 ml. of N- H_2SO_4 , the volume was brought to 5 ml. with water. About 3 ml. of dithizone (0.0012%) in carbon tetrachloride was added. The tubes were then shaken for 10 min. Extinction values were determined at 510 m μ , the absorption minimum of free dithizone.

Magnesium was estimated with Titan yellow according to Orange & Rhein (1951) except that the final volume was 2.5 ml.

Other experimental procedures used are described in previous papers (Appleby & Morton, 1954, 1959).

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RESULTS

Reaction of yeast lactic dehydrogenase with ferricyanide, cytochrome c and methylene blue

With 0.83 mM-potassium ferricyanide at pH 8.0, the rate of reduction appeared to be linear with time (Fig. 1, A). This suggests that the reaction is of zero order. However, a first-order reaction may be observed under other conditions.

With approx. 0.03 mM-cytochrome *c* (heart muscle) at pH 7.4, the rate of reaction decreased with time (Fig. 1, B). The plot of log (ferricytochrome *c* at zero time/ferricytochrome *c* remaining at time *T*) against *T* is linear (Fig. 1, B). Thus under these conditions the reduction of cytochrome *c* is a first-order reaction. The amount of cytochrome *c* reduced in the first minute was used as the estimate of the rate with this compound in routine assays.

In early studies with both crude and crystalline enzyme and with 0.02–0.03 mM-ferricytochrome *c*, the rate of reaction was apparently proportional to the amount of enzyme, up to that which produced a change of extinction at 549.5 m μ (see Appleby & Morton, 1959) of about 0.14 during the first minute. The specific activity so obtained with 0.02–0.03 mM-ferricytochrome *c* at 18–20° at pH 7.0 was 1955 units/mg. However, with 0.05 mM-cytochrome *c* (heart muscle) and with the assay system of Boeri, Cutolo, Luzzati & Tosi (1955), the specific activity of the crystalline enzyme at 20° was found to be 5870 units/mg. (i.e. approx. 2940 μ moles of lactate oxidized/hr./mg. of enzyme). This greater activity is due to the higher concentration of cytochrome *c* and to the protective effect of ethylenediaminetetra-acetate (EDTA) used in the assay system.

The rates of the reactions with ferricyanide, heart-muscle cytochrome *c* and methylene blue at

Fig. 1. Reduction of ferricyanide (A) and of heart-muscle ferricytochrome *c* (B), catalysed by yeast lactic dehydrogenase. —, Extinction against time (*T*); - - -, log (*a/b*) against time (*T*), where *a* is the concentration of oxidized acceptor at zero time, and *b* is the concentration remaining at time (*T*). (For conditions see text.)

Table 1. *Temperature coefficient and activation energy of the enzymically catalysed oxidation of lactate by ferricyanide, cytochrome c and methylene blue*

Twice-recrystallized enzyme was used. The rates of reduction of ferricyanide and of cytochrome *c* were measured spectrophotometrically with the constant-temperature cell holder of Dixon (1954); that of methylene blue was measured visually with Thunberg tubes held in a constant-temperature glass water bath. The methods are described by Appleby & Morton (1959). All assay mixtures contained mM-EDTA.

Hydrogen acceptor	Assay system				Temperature coefficient (Q_{10}) and activation energy (μ)			
	Concn. of hydrogen acceptor (mM)	Buffer	pH	Temp.	Activity (units/ml.)	Temp. range	Q_{10} (kcal./mole)	μ
Potassium ferricyanide	0.83	0.03 M-sodium pyrophosphate-HCl	8.0	20°	452	20–30°	1.5	7.2
				30	675			
Heart-muscle cytochrome <i>c</i>	0.05	0.05 M-sodium phosphate-HCl	7.0	20	342	20–30	1.5	7.6
				30	518			
Methylene blue	0.08	0.06 M-sodium acetate	5.2*	20	74	20–38	1.6	8.7
				38	170			

* This pH is not the optimum for this reaction: see Appleby & Morton, 1959.

two different temperatures are shown in Table 1, together with the temperature coefficients (Q_{10}) for these reactions. The Arrhenius equation as given by Sizer (1943) was used with these measurements to calculate the activation energies shown in Table 1.

The optimum pH values for the reaction catalysed by a crude preparation of enzyme with ferricyanide, heart-muscle cytochrome c and

Fig. 2. Effect of pH on the rate of reduction of various hydrogen acceptors catalysed by a crude preparation of yeast lactic dehydrogenase. Results with 0.83 mM-potassium ferricyanide (curve A), heart-muscle ferricytochrome c (0.02 mM) (curve B) and 0.08 mM-methylene blue (curve C).

Fig. 3. Effect of pH on the rate of reduction of various hydrogen acceptors catalysed by crystalline yeast lactic dehydrogenase. Other details are as described for Fig. 2.

methylene blue were 8.5, 6.0 and 5.2, respectively (Fig. 2). However, with the same acceptors, the pH optima were 8.0, 7.0 and 6.8 for crystalline enzyme (Fig. 3, see also, Appleby & Morton, 1959).

Reaction of yeast lactic dehydrogenase with oxygen

As observed by Bach *et al.* (1946), cytochrome b_2 is slowly auto-oxidizable. Freshly prepared crystals containing about 0.7% of sodium lactate were rapidly dissolved in a solution at 0° and at pH 7.4, previously equilibrated with air and containing 0.5M-NaCl, 0.1 mM-EDTA and 0.01M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer. Spectroscopic observations showed that the cytochrome was completely oxidized in 2 min. Under these conditions, at pH 7.4 and at 0°, approx. 10 μ moles of lactate were oxidized/hr./mg. of enzyme (approx. 80 moles of lactate/hr./mole of enzyme). At pH 10.0 and at 15° auto-oxidation appeared to be much more rapid. At pH values less than 5.5, at which the enzyme is relatively slightly soluble, slow auto-oxidation as well as denaturation of the enzyme (see Appleby & Morton, 1959) was observed.

Substrate specificity of yeast lactic dehydrogenase

As shown in Table 2, of a range of salts of hydroxy acids, the crystalline enzyme reacted with only glycollate, lactate and α -hydroxy- n -butyrate. The highest activity was obtained with lactate itself.

The crystals had no diaphorase activity. No reduction of dye was observed in a system containing (in 3 ml.) 2 μ moles of reduced diphosphopyridine nucleotide, 0.15 μ mole of 2:6-dichlorophenol-indophenol, 0.03M-sodium pyrophosphate-acetic acid buffer, pH 8.5, and enzyme.

Table 2. *Substrate specificity of yeast lactic dehydrogenase*

A small pellet of freshly prepared, twice-crystallized enzyme was dissolved in a de-aerated solution at pH 6.8 and at 0° containing 0.5M-NaCl and 0.1 mM-EDTA. The rate of reduction of potassium ferricyanide, in a solution containing 0.03M-sodium pyrophosphate-HCl buffer, pH 8.0, mM-EDTA, 0.1M-hydroxy acid (as the sodium salt) and either 0.01 or 0.1 ml. (as appropriate) of the solution of enzyme, was measured as previously described (Appleby & Morton, 1954, 1959). Activities are expressed as ferricyanide units/ml.

Substrate	Activity
None	0
Glycollate	144
DL-Lactate	2990
DL- α -Hydroxy- n -butyrate	1205
DL- β -Hydroxy- n -butyrate	0
α -Hydroxy- <i>iso</i> -butyrate	0

Absorption spectrum of crystalline cytochrome b_2

Fresh crystals or solutions of reduced yeast lactic dehydrogenase are pink, and change to red-brown on auto-oxidation. The absorption spectra of oxidized and lactate-reduced enzyme at pH 6.8 are shown in Fig. 4. The maxima at 556.5 and 528 $m\mu$ in the reduced enzyme correspond with the positions of the α - and β -bands of cytochrome b_2 as described by Bach *et al.* (1946). In addition, maxima occur at 424 and 330 $m\mu$ (γ - and δ -bands) and at 265 $m\mu$. The values of ϵ at these maxima are given in Table 9.

Fig. 4. Absorption spectra of riboflavin phosphate and of oxidized and lactate-reduced cytochrome b_2 (yeast lactic dehydrogenase). A Beckman spectrophotometer, model DU, was used and measurements were made in an atmosphere of nitrogen with Thunberg-type cuvettes with a light-path of 1 cm. (see Appleby & Morton, 1959). For curve A twice-crystallized enzyme was dissolved in m -sodium lactate, pH 6.8, and diluted in 0.1 M -sodium pyrophosphate, pH 6.8; the maxima are at 556.5, 528, 424, 330 and 265 $m\mu$. For curve B, the preparation was dialysed against 0.1 M -sodium pyrophosphate-HCl buffer, pH 6.8, at 0° to remove lactate and then rapidly oxidized by aeration; the maxima are at 413, 359 and 265 $m\mu$. In each case, the reference cuvette contained the appropriate buffer system, with or without lactate as necessary. For curve C, oxidized riboflavin phosphate, at a concentration equivalent to that of the flavin in the cytochrome b_2 , was dissolved in 0.1 M -sodium pyrophosphate-HCl, pH 6.8; the maxima are at 445, 372 and 265 $m\mu$. The extinction coefficient (ϵ) is given for a molar concentration of enzyme-bound haem. A, Lactate-reduced cytochrome b_2 ; B, oxidized cytochrome b_2 ; C, oxidized riboflavin phosphate.

The effect of pH on the absorption spectrum of the lactate-reduced cytochrome b_2 was investigated. At pH 5.0 there was no change from that found at pH 6.8, but at pH 10.0 an additional shoulder appeared near 450 $m\mu$. This shoulder may be due to the presence of dissociated oxidized flavin.

It was observed spectroscopically that at the temperature of liquid air (see Keilin & Hartree, 1949) the visible absorption bands of the lactate-reduced cytochrome b_2 at pH 6.8 were considerably intensified. The α -band was shifted to 555.2 $m\mu$ (see Appleby & Morton, 1954), but no splitting of this band was detected, in contrast with the observation of other workers (Chance, Klingenberg & Boeri, 1956; Lindenmayer & Estabrook, 1958) with non-crystalline preparations of cytochrome b_2 .

At pH 6.8 the oxidized enzyme has a para-haematin-type spectrum, with general absorption at 530–560 $m\mu$, and with maxima at 413 and 359 $m\mu$ (Fig. 4). In the ultraviolet region, the absorption spectrum resembles that of the lactate-reduced enzyme.

Components of crystalline cytochrome b_2

Protohaem. Spectroscopic and spectrophotometric measurements of the reduced pyridine haemochromogen formed from crystalline lactic dehydrogenase showed a maximum (α -band) at 556 $m\mu$, thus confirming the observations of Bach *et al.* (1942). The similar haemochromogens formed from crystalline protohaemin, and also from crystalline haemoglobin under identical conditions, showed maxima at 556.5 and 556 $m\mu$, respectively.

For the reduced pyridine haemochromogen from cytochrome b_2 , the maximum extinction of the α -band, and the ratio of this value to the minimum extinction near 540 $m\mu$, was found to depend on the method of formation. When the enzyme was dissolved in the alkali before the addition of pyridine, the ratio of ($E_{\max}$ near 556 $m\mu$)/($E_{\min}$ near 540 $m\mu$) was 2.5 or less. When the pyridine was added to an aqueous suspension of freshly prepared or of freeze-dried crystals before the addition of NaOH and $Na_2S_2O_4$, the ratio was 3.45. For the reduced pyridine haemochromogen formed from crystalline protohaemin, Paul, Theorell & Åkeson (1953) obtained a ratio of 3.46, and ϵ for the α -band was 34.2×10^3 ; in the present work, with crystalline protohaemin (8.2% of iron), the values obtained were 3.43, and 33.2×10^3 , respectively.

Thus protohaem is the only haem present in crystalline yeast lactic dehydrogenase, which is a true cytochrome.

From the results obtained by adding NaOH and $Na_2S_2O_4$ to the enzyme in pyridine to form the reduced pyridine haemochromogen, and with the value for ϵ at 556 $m\mu$ of Paul *et al.* (1953), the

Table 3. *Haem, flavin and metal components of crystalline yeast lactic dehydrogenase*

Analyses were carried out on different batches of enzyme recrystallized at least twice. Methods used are described in the text. The proportion of component relative to haem is expressed as mole (or g.atom) of component/mole of haem.

Component	Assay procedure	Amount (%)	Proportion of component relative to haem	Wt. of enzyme/mole (or g.atom) of component estimated
Protohaem	Pyridine haemochromogen	0.77	(1.0)	80 000
Riboflavin phosphate	Fluorimetry (Bessey, Lowry & Love, 1949)	0.5	1.1	73 400
	Extinction at 445 m μ of			
	(1) heated extract		1.0	82 500
	(2) HClO ₄ extract		1.0	80 500
Total iron	<i>o</i> -Phenanthroline complex (Drabkin, 1941)	0.091	1.3	61 400
Non-haem iron	By difference from haem		0.3	—
Copper	Sandell (1944)	0.016	0.2	—
Magnesium	Orange & Rhein (1951)	0.02	0.65	—

enzyme contains 0.77 % of haem. Hence the weight/mole of haem is approx. 80 000 g. (Table 3).

Riboflavin phosphate. When irradiated at about 360 m μ with a Hanovia lamp, solutions of freshly prepared enzyme did not fluoresce, whereas an intense yellow-green fluorescence, characteristic of free oxidized flavins, appeared after denaturation by either heat or acid. A solution of enzyme was extracted with phenol according to Crammer (1948), and the extract was examined by paper chromatography as described by Whitby (1950). Only riboflavin phosphate was detected. As determined by the fluorimetric method of Bessey, Lowry & Love (1949), only riboflavin phosphate (or a flavin of similar heat stability and solubility properties) was present; the amount (0.5 %) indicated a weight/mole of flavin of 73 400 g. (Table 3). Flavin was also estimated by measuring the total absorption at 445 m μ of the oxidized supernatant remaining after precipitation of the haem-protein both by heat denaturation and with perchloric acid. From these results (Table 3) a weight/mole of flavin of 82 500 g. and 80 500 g. respectively was obtained. Within the experimental error of the various determinations therefore, the protohaem and riboflavin phosphate are present in equimolecular proportions in the crystalline enzyme, which has a minimum molecular weight of about 80 000.

Iron and other metals. The results obtained with a spark spectrograph (see Methods section) showed that only iron, copper and magnesium were present in the crystalline enzyme in significant amounts (Table 4). These metals were therefore determined quantitatively by colorimetric methods as described in the Methods section.

The total iron content of an enzyme preparation crystallized twice was 0.091 %, corresponding to

Table 4. *Metal components of different preparations of crystalline yeast lactic dehydrogenase*

Twice-recrystallized enzyme was washed with 0.05 M-sodium lactate and the crystals were packed by centrifuging and then drained and freeze-dried. The material so obtained contained less than 1 % of lactate. It was placed directly in the hollow lower electrode of a spark spectrograph. The relative amount of each metal was estimated from photographs of the spark spectra.

Relative amount of metal	Metal components in	
	Preparation 1* (enzyme of normal activity)	Preparation 2* (enzyme of low activity)
Trace to strong trace	Si†	Cu
Trace	Cu, Mg	Fe, Na, Mg
Faint trace to trace	Fe	Zn
Faint trace	Ca	Ca
Very faint trace to faint trace	Al	—
Very faint trace	Zn, Pb	Ag
Very faint trace (doubtful)	Ag	—
Not detected	Mo, Co, Ni	Mo, Co, Ni, Al

* The activity of preparation 1 was approx. 6000 and that of preparation 2 was 2500 ferricyanide units/mg.

† A glass spatula was used to scrape this sample from the centrifuge tube.

1.3 g.atoms of iron/mole of enzyme haem. Thus the enzyme contains only 0.3 g.atom of non-haem iron/mole (Table 3).

A preparation with a specific activity of 6300 ferricyanide units/mg. contained 0.016 % of copper and 0.02 % of magnesium, corresponding to 0.2 and 0.65 g.atom of copper and magnesium respectively/mole of haem (Table 3).

Deoxyribose polynucleotide. The absorption at about 265 m μ (Fig. 3) in the crystalline enzyme is

due to riboflavin phosphate and a deoxyribose polynucleotide (Appleby & Morton 1954; Morton 1955; Appleby 1957). The isolation and identification of the polynucleotide (Appleby 1957) will be described in a subsequent paper.

Instability of yeast lactic dehydrogenase

The considerable instability of purified preparations of the enzyme was noted by Bach *et al.* (1946). It was observed during the development of methods for purification of the enzyme (see Appleby & Morton, 1959) that preparations of specific activity greater than 500 ferricyanide units/mg. rapidly lost activity, even when stored at 0°. There was less decline in activity if the enzyme was kept in the presence of excess of substrate, and further improvement was obtained by maintaining the enzyme under anaerobic conditions. These observations suggested that the loss of activity was due to hydrogen peroxide formed by auto-oxidation of the enzyme. It was found that hydrogen peroxide is indeed inhibitory, and that some protection was obtained with catalase (Table 5).

However, since anaerobic conditions reduced, but did not prevent, the loss of activity, it was apparent that inactivation was also caused by factors other than hydrogen peroxide. A solution of once-crystallized enzyme of specific activity 6620 ferricyanide units/mg. was dissolved in 0.03M-sodium pyrophosphate containing 0.1M-sodium lactate, pH 6.8, and dialysed anaerobically against 0.05M-sodium lactate, pH 6.8, at 0°. Crystals appeared in 1 hr. but dialysis was continued for 5 days. The

specific activity of the crystals so obtained was only 3920 ferricyanide units/mg. On irradiation, the mother liquor of these crystals and the diffusate both showed a strong yellow-green fluorescence, characteristic of free, oxidized flavin. Addition to the enzyme of substantial amounts of either cysteine-reduced riboflavin phosphate, or of cystein-reduced flavinadenine dinucleotide, failed to reactivate the preparation. The addition of 0.01M-riboflavin phosphate to the 0.05M-sodium lactate before dialysis (see Appleby & Morton, 1959) did not improve the yield or the specific activity of later preparations of crystals. Hence it appeared that the dissociation of riboflavin phosphate from the enzyme was irreversible.

There appeared to be a relationship between the loss of activity during storage, and the copper content of the crystalline enzyme. One preparation had a specific activity of 5500 ferricyanide units/mg. as determined immediately after dissolving the crystals in 0.5M-sodium lactate at pH 6.8; the activity fell to 3800 units/mg. after holding aerobically for 1 hr. at 0°, and to 2500 units/mg. after further storage. At this stage the solution showed a strong flavin fluorescence when irradiated. As estimated with a spark spectrograph, this preparation of crystals had a high content of copper (Table 4, preparation 2). As shown in Table 6, the addition of mM-copper sulphate to a different preparation of crystalline enzyme caused almost complete inactivation after aerobic storage for 18 hr. at 0°. This inactivation was considerably reduced by addition of EDTA (Table 6), as was also the loss of activity caused by aerobic storage of enzyme in the absence of added Cu²⁺ ions (Table 7). The addition of EDTA (about 0.1 mM) also considerably reduced the rate of inactivation of enzyme stored under anaerobic conditions.

The following observations support the finding that the metal-catalysed auto-oxidation of cytochrome *b₂* is accompanied by dissociation of riboflavin phosphate. A pellet of lactate-reduced crystals was freshly prepared in the absence of EDTA. The pellet was pink and did not fluoresce on irradiation. After exposure to air at 0° the colour changed to orange, as oxygen diffused through the pellet. The well-defined front of the orange oxidized cytochrome corresponded exactly with the zone of yellow-green fluorescence as seen on irradiation of the crystals. However, a similar pellet of crystals prepared in the presence of 0.1 mM-EDTA showed only faint fluorescence in the zone of oxidized cytochrome.

Prosthetic groups of yeast lactic dehydrogenase

Riboflavin phosphate. The observations cited above indicate that protein-bound riboflavin phosphate is essential for enzymic activity and

Table 5. *Effects of catalase and of added hydrogen peroxide on the activity of yeast lactic dehydrogenase*

A solution of once-crystallized enzyme (approx. 40 µg./ml.) in M-sodium lactate, pH 6.8, was used. About 100 µg. of crystalline liver catalase (Armour Laboratories, U.S.A.)/ml. was added to a portion of the enzyme solution. The solutions were stored aerobically. The rate of reduction of potassium ferricyanide in 0.03M-sodium pyrophosphate-acetic acid buffer, pH 8.0, was measured as previously described (Appleby & Morton, 1954, 1959). Activities are expressed as ferricyanide units/ml.

Stage	Storage and treatment conditions	Enzymic activity	
		With catalase	Without catalase
1	Fresh preparation	248	248
2	Held for 4 days at 0°	230	94
3	Approx. 0.2 mM-H ₂ O ₂ was added to material treated as described above, then held for 10 min. at 0°	198	78
4	H ₂ O ₂ increased to mM concentration; enzyme was then held for 10 min. at 0°	184	29.5

Table 6. *Inactivation of yeast lactic dehydrogenase by Cu^{2+} ions and protection by ethylenediaminetetra-acetate*

A solution of enzyme (crystallized once) in 0.5M-sodium lactate, pH 6.8, was used. All tubes, except the controls, were aerated. In each experiment, oxygen was displaced from the control tube with nitrogen. The rate of reduction of ferricyanide was estimated (Appleby & Morton, 1954, 1959) with 0.03M-sodium pyrophosphate-HCl, pH 8.0, and at 18°. Activities are expressed as ferricyanide units/ml.

In Expt. 1, copper sulphate was added, and in Expt. 2, copper sulphate and EDTA were added, to give the concentration shown. All tubes were stored for 18 hr. at 0°.

Expt. 1			Expt. 2			
Concn. of CuSO_4 (μM)	Enzymic activity		Concn. of CuSO_4 (μM)	Concn. of EDTA (μM)	Enzymic activity	
	Anaerobic storage	Aerobic storage			Anaerobic storage	Aerobic storage
0	224	186	0	0	179	119
0.1	—	167	10	0	—	10
10	—	57	10	1	—	10
1000	—	2	10	10	—	97
			10	100	—	195

Table 7. *Protection of yeast lactic dehydrogenase by ethylenediaminetetra-acetate*

A preparation of once-crystallized enzyme was dissolved in 0.5M-sodium lactate, pH 6.6, and EDTA was added to give the concentrations shown below. Activity with ferricyanide (Appleby & Morton, 1954, 1959) was determined with 0.03M-sodium pyrophosphate-HCl buffer, pH 8.0, at 18°, immediately and after holding the preparation aerobically for 18 hr. at 0°. Activities are expressed as ferricyanide units/ml.

Concn. of EDTA (μM)	Enzymic activity	
	At commencement	After storage (18 hr.)
0	366	80
0.1	370	258
1	—	262
10	383	320
100	460	465

suggest that riboflavin phosphate acts as a hydrogen acceptor in the oxidation of lactate.

With oxidized lactic dehydrogenase, the intense Soret band due to the haem group (Fig. 4, curve *B*) prevents observation of the expected absorption bands at about 375 and 450 $\text{m}\mu$ due to oxidized riboflavin phosphate. For the same reason, the difference spectrum (oxidized minus reduced enzyme) is not that of a typical flavoprotein. However, for the lactate-reduced enzyme, ϵ at 450 $\text{m}\mu$ is only 6.2×10^3 (Fig. 4, curve *A*). If the riboflavin phosphate was not reduced by lactate, the minimum value of ϵ expected at this wavelength, due to oxidized riboflavin phosphate, would be 11.2×10^3 (Fig. 4, curve *C*). Hence the flavin group must be at least partly reduced by lactate. This is supported by the following observations. A freshly prepared pellet of crystalline enzyme was dissolved in 0.5M-NaCl containing 0.1 mM-EDTA, pH 6.8, at 0°. Measurements of the visible absorption bands were

made with a Hartridge reversion spectroscope as the solution was gently shaken in the air. The bands of the reduced enzyme at about 556.5 and 527 $\text{m}\mu$ slowly faded and were replaced by broad but weak absorption bands at approx. 567 and 533 $\text{m}\mu$. These bands disappeared completely on further oxidation. The bands at 556.5 and 527 $\text{m}\mu$ reappeared immediately on the addition of a small amount of lactate. The bands at 567 and 533 $\text{m}\mu$ are not seen in the fully oxidized enzyme, which has a parahaematin-like spectrum (Fig. 4, curve *B*), and they are observed only momentarily as the fully reduced enzyme is oxidized. Hence they are the absorption bands of the enzyme at an intermediate state of oxidation. As far as is known (see Morton, 1958), no other cytochromes show similar changes during oxidation of the reduced compound. However, Beinert (1957) reported that some flavoprotein enzymes have a single absorption band near 560 $\text{m}\mu$ which he ascribed to the semi-quinoid form of the flavin prosthetic group. Although Beinert (1957) did not report any band near 533 $\text{m}\mu$, it seems probable that the bands at 567 and 533 $\text{m}\mu$ indicate the formation of a semi-quinone of riboflavin phosphate during oxidation of lactic dehydrogenase and hence the participation of flavin in the enzymic reaction.

Storage of oxidized enzyme at 0° in the absence of EDTA caused rapid loss of activity, as measured by reduction of ferricyanide, and, correspondingly, a decline in the amount of haem capable of reduction by lactate on incubation for about 10 min. at 0° (Table 8). However, there was little change in the amount of haem reducible with $\text{Na}_2\text{S}_2\text{O}_4$ (Table 8). The rate of reduction of ferricyanide declined about twice as rapidly as did the amount of haem reducible by lactate in 10 min. at 0°. The sample stored for 18 hr. (Table 8) showed a strong greenish fluorescence on irradiation, indicating

Table 8. *Effects of aerobic storage on the reduction of the haem group of yeast lactic dehydrogenase by lactate and by dithionite*

Freshly recrystallized enzyme was suspended in water at 0° and held aerobically at 0°. At the times indicated, portions (0.1 ml.) were diluted 50-fold in 0.5M-sodium lactate containing 0.1 mM-EDTA, pH 6.8, at 0°. The rate of reduction of ferricyanide (in 0.03M-sodium pyrophosphate-HCl buffer, pH 8.0, containing 0.1 mM-EDTA) was measured immediately. Measurements were made of the position and height of the Soret band, and of the extinction at 424 m μ , at approx. 10 min. after preparation of the dilution and after the further addition of about 2 mg. of Na₂S₂O₄ to the cuvette (capacity 3 ml.) containing the diluted enzyme. It was assumed that the cytochrome was fully reduced by Na₂S₂O₄ and the proportion of the haem reduced by lactate was calculated by assuming the ratio of extinction values at 424 m μ for oxidized enzyme/reduced enzyme to be 0.354 (see Fig. 4). The extinction values are given for the undiluted preparation.

Activities are expressed as ferricyanide units/ml. and wavelengths are in m μ .

Storage time at 0°	Activity (units/ml.)	Relative activity after storage (%)	Extinction values for Soret band						Proportion of haem group reduced by lactate (%)
			Reduced with lactate			Reduced with Na ₂ S ₂ O ₄			
			$\lambda_{\max.}^*$ (m μ)	<i>E</i>		$\lambda_{\max.}^*$ (m μ)	<i>E</i> _{424 mμ}		
				At $\lambda_{\max.}$	At 424 m μ				
0	23800	(100)	424	11.5	11.5	424	11.0	(105)	
10 min.	13210	55	423.5	10.1	10.1	424	11.0	87	
18 hr.	5050	21	419	7.4	6.5	424	10.7	43	

* $\lambda_{\max.}$ indicates the wavelength at which the maximum extinction of the Soret band was obtained.

some dissociation of the flavin group. These observations support the suggestion (Appleby & Morton, 1954) that riboflavin phosphate, and not protohaem, acts as the primary acceptor of hydrogen atoms from lactate.

Protohaem. It was observed spectroscopically that the addition of ferricyanide to a dilute solution of cytochrome *b*₂ in excess of lactate caused the immediate disappearance of the α - and β -bands of the reduced enzyme. When all the ferricyanide was reduced, these bands reappeared. They disappeared finally when the lactate was completely oxidized. Hence the haem group undergoes reversible oxidation and reduction during the oxidation of lactate.

Numerous attempts to separate lactic dehydrogenase activity from cytochrome *b*₂ were unsuccessful (Appleby & Morton, 1954, 1959).

DISCUSSION

The crystallization of cytochrome *b*₂ and identification of riboflavin phosphate and protohaem as the prosthetic groups was first reported by Appleby & Morton (1954). The evidence described here and earlier (Appleby & Morton, 1954, 1959; Appleby, 1957) establishes that cytochrome *b*₂ is identical with yeast lactic dehydrogenase and that the enzyme contains flavin and haem in equimolecular proportions. This finding has since been confirmed by other workers (Boeri *et al.* 1955; Boeri & Tosi, 1956; Nygaard, 1958) with non-crystalline enzyme preparations.

Yamashita *et al.* (1957) have described a crystalline preparation of a yeast haemoprotein which

these workers called 'cytochrome *b*₂' but which had no lactic dehydrogenase activity. Bach *et al.* (1946) first observed that preparations in which the haem group was reducible by dithionite, but not by lactate, could be obtained by denaturation of yeast lactic dehydrogenase. Loss of enzymic activity, accompanied by the appearance of fluorescence due to free flavin, was frequently observed by us during investigation of chromatographic methods for purification of the enzyme (see also, Nygaard, 1958). Moreover, when we subjected crystalline lactic dehydrogenase to chromatographic procedures rather similar to those of Yamashita *et al.* (1957), enzymic activity was lost and free flavin (not flavoprotein) was detected in the eluates. In addition to protein denaturation, metal-catalysed oxidation causes rapid dissociation of the flavin group of the enzyme accompanied by loss of enzymic activity (Tables 6–8); however, the haem group remains reducible by dithionite (Table 8). In view of the use of autolysis for 1 week at pH 8.5 during purification (Yamashita *et al.* 1957), it is not surprising that the haemoprotein obtained by these workers does not contain riboflavin phosphate (Yamanaka, Horio & Okunuki, 1958). The material isolated by Yamashita *et al.* (1957) thus appears to be a product derived from the native flavohaemoprotein, cytochrome *b*₂. Our work gives no support for the suggestion of Yamanaka *et al.* (1958) that the crystalline lactic dehydrogenase of Appleby & Morton (1954, 1959) is a complex of a haemoprotein ('cytochrome *b*₂') and a flavin enzyme.

Table 9 compares the properties of the crystalline lactic dehydrogenase of Appleby & Morton (1954,

Table 9. *Comparative properties of several preparations of yeast lactic dehydrogenase.*

In each case the values refer to the preparation of maximum purity and to enzymic activities determined under optimum conditions as defined by the various authors. Wavelengths are given in $m\mu$: the extinction coefficient (ϵ) in each case is given for a molar concentration of enzyme-bound haem.

Property	Values according to authors indicated		
	Appleby & Morton (1954, 1959 and this paper)	Boeri, Cutolo, Luzzati & Tosi (1955) and Boeri & Tosi (1956)	Bach, Dixon & Zerfas (1946)
Chemical and physical properties:			
Weight of enzyme (g.)/mole of haem	80 000	230 000	850 000
No. of prosthetic groups/minimum weight of enzyme:			
Protohaem	1	1	1
Riboflavin phosphate	1	1	—
Non-haem iron	0*	8	—
Deoxyribose nucleotide	15†	—	—
Principal absorption bands of reduced enzyme:			
α -Band $\left\{ \begin{array}{l} \lambda \\ 10^{-3} \epsilon \end{array} \right.$	$\left\{ \begin{array}{l} 556.5 \\ 38.8 \end{array} \right.$	$\left\{ \begin{array}{l} 557 \\ 36 \end{array} \right.$	$\left\{ \begin{array}{l} 556.5 \\ 32.6 \end{array} \right.$
β -Band $\left\{ \begin{array}{l} \lambda \\ 10^{-3} \epsilon \end{array} \right.$	$\left\{ \begin{array}{l} 528 \\ 18.6 \end{array} \right.$	$\left\{ \begin{array}{l} 528 \\ 25.4 \end{array} \right.$	$\left\{ \begin{array}{l} 530 \\ 22.2 \end{array} \right.$
γ -Band $\left\{ \begin{array}{l} \lambda \\ 10^{-3} \epsilon \end{array} \right.$	$\left\{ \begin{array}{l} 424 \\ 232 \end{array} \right.$	$\left\{ \begin{array}{l} 423 \\ 224 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ — \end{array} \right.$
δ -Band $\left\{ \begin{array}{l} \lambda \\ 10^{-3} \epsilon \end{array} \right.$	$\left\{ \begin{array}{l} 330 \\ 52.2 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ — \end{array} \right.$	$\left\{ \begin{array}{l} — \\ — \end{array} \right.$
Ultraviolet-band $\left\{ \begin{array}{l} \lambda \\ 10^{-3} \epsilon \end{array} \right.$	$\left\{ \begin{array}{l} 265 \\ 199 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ — \end{array} \right.$	$\left\{ \begin{array}{l} — \\ — \end{array} \right.$
Enzymic activities:			
With heart-muscle cytochrome <i>c</i> at 20°			
pH optimum	7.0	7.0-8.0	—
μ moles reduced/hr./mg. of enzyme	5 870	3 660	—
moles reduced/min./mole of enzyme-bound haem	7 850	14 000‡	—
With potassium ferricyanide at 20°			
pH optimum	8.0	7.0-8.0	—
μ moles reduced/hr./mg. of enzyme	7 750	3 660	—
moles reduced/min./mole of enzyme	10 350	14 000‡	—
With methylene blue at 38°			
pH optimum	6.8	5.8	5.3
μ moles reduced/hr./mg. of enzyme	2 000	580	—
moles reduced/min./mole of enzyme-bound haem	2 700	2 200	—
$Q_{MB}^{\S}$	56 800	—	3000

* Actual values of 0.1 (unpublished studies of J. McD. Armstrong & R. K. Morton) to 0.3 g.atoms of non-haem iron/mole of haem (Table 3) are regarded as indicating slight impurity only.

† Appleby (1957).

‡ These high values reflect the low haem iron found by Boeri *et al.* (1955).

§ As defined by Bach *et al.* (1946).

1959) with those of the non-crystalline enzyme preparations of other workers (Bach *et al.* 1946; Boeri *et al.* 1955). Electrophoretic and ultracentrifuge studies, and amino acid analyses, which will be described in subsequent papers, provide further evidence of the homogeneity of the crystalline enzyme of Appleby & Morton (1954, 1959). The weight of the crystalline enzyme/mole of haem or of riboflavin phosphate (about 80 000 g.) is rather similar to the weight/mole of flavin of a number of the purified flavoprotein enzymes, such as 'old yellow enzyme' of yeast (52 000 g.; Theorell, 1935;

Theorell & Åkeson, 1956), reduced triphosphopyridine nucleotide-cytochrome *c* reductase of yeast (75 000 g.; Haas, Horecker & Hogness, 1940) and D-amino acid oxidase of kidney (69 000 g.; Blanchard, Green, Nocito & Ratner, 1944). On the basis of the reaction of yeast lactic dehydrogenase with cytochrome *c*, Green & Beinert (1955) predicted that the enzyme contains a metal chelated by the flavin group. However, the analyses in Tables 3, 4 and 9 show that there is insufficient non-haem iron and copper, and insufficient of any other likely metal, for a function in mediating

electron transfer as proposed by Green & Beinert (1955). Boeri & Tosi (1956) propose that the 8 g.-atoms of non-haem iron in the lactic dehydrogenase of Boeri *et al.* (1955) (see Table 9) have an essential function in mediating reactions with cytochrome *c* (cf. Mahler & Green, 1954; Mahler & Elowe, 1954). However, the results presented here and in summary earlier (Morton, 1955) strongly suggest that the non-haem iron found by Boeri & Tosi (1956) is an impurity of the preparation. Moreover, we obtained no stimulation of the activity with cytochrome *c* by addition of ferrous sulphate to the crystalline enzyme (Appleby, 1957). It is considered that the magnesium (approx. 1 g./atom/mole of haem; Table 4) is probably present in non-specific association with the polynucleotide component of the cytochrome (see Table 9).

The extinction values for the principal absorption bands of crystalline cytochrome b_2 (Fig. 4 and Table 9) are greater than those of any similar cytochromes as yet purified (see Morton, 1958, Table 5). By spectrophotometric titration with lactate of a non-crystalline preparation of cytochrome b_2 (Boeri *et al.* 1955), ϵ for the reduced minus oxidized preparation at 557 $m\mu$ was estimated by Chance *et al.* (1956) as 21×10^3 ; the value at pH 6.5–6.8 for the crystalline enzyme is 23.9×10^3 (see Fig. 4). The quotient $E_{556.5\ m\mu}/E_{550\ m\mu}$ for crystalline cytochrome b_2 of Appleby & Morton is 1.8–1.9; for the preparation of Bach *et al.* (1946) it is 1.6; and for that of Boeri *et al.* (1955) it is 1.4. As higher values generally reflect a greater purity, it would appear that the preparation of Boeri *et al.* (1955) may contain some cytochrome *c* or cytochrome c_1 or similar type of impurity. Table 9 shows that the specific activity (μ moles of acceptor reduced/hr./mg. of enzyme) of the preparation of Appleby & Morton is very much higher than that of Boeri *et al.* (1955) with all acceptors. However, the activity as moles of cytochrome *c* reduced/min./mole of haem is greater for the preparation of Boeri *et al.* (1955). This may indicate difficulties in estimation of proto-haem or of enzymic activity, or the presence in the cruder preparation of factors such as yeast cytochrome *c* or c_1 , which stimulate the activity with heart-muscle cytochrome *c*.

As shown by Table 2, glycollate and α -hydroxybutyrate, as well as lactate, are substrates of the enzyme. It has also been shown by J. McD. Armstrong & R. K. Morton (unpublished work) that certain other hydroxy acids, including L-malic acid and DL-glyceric acid, and also reduced di- and triphosphopyridine nucleotide, are not substrates of the crystalline enzyme. Contrary findings by other workers (Yamashita *et al.* 1957; Yamanaka *et al.* 1958; Yamashita, Horio & Okunuki, 1958) must be attributed to contaminating enzymes in the preparations used. Dickens & Williams (1956)

recognized that the oxidation of L-glyceric acid by a preparation of yeast lactic dehydrogenase according to Boeri *et al.* (1955) was possibly due to an enzymic impurity.

The enzyme is specific for the L-isomer of lactate (Mahler & Huennkens, 1953; Boeri *et al.* 1955). The oxidation of L-lactate is not inhibited by D-lactate (Boeri *et al.* 1955) so that DL-lactate is suitable as a substrate for enzymic studies.

Several of the findings described here relate to the function of riboflavin phosphate in cytochrome b_2 (Appleby & Morton, 1954). Inactivation of the enzyme accompanied by dissociation of the flavin prosthetic group is caused by oxidation by hydrogen peroxide or by Cu^{2+} ions under aerobic conditions (Tables 5, 7 and 8). Whereas the enzyme is strongly inhibited by *p*-chloromercuribenzoate (0.1–10.0 mM) (Appleby & Morton, 1954; Boeri *et al.* 1955), moniodoacetate (10 mM) under similar conditions had little effect on the activity (Boeri *et al.* 1955). This suggests that the thiol groups of the enzyme are masked or bound (see Barron, 1951). These several observations are in accord with the hypothesis that the flavin is linked to the apoenzyme by a bond between the imino group of the isoalloxazine of the riboflavin phosphate (see Theorell, 1955) and the thiol group of a cysteine residue of the apoenzyme. Oxidation of the enzyme and partial dissociation of the oxidized riboflavin phosphate (see Vestling, 1955) would expose the –SH groups. Metal-catalysed oxidation of the –SH groups would thus lead to irreversible displacement of the riboflavin phosphate, as observed. The protective effect of ethylenediaminetetra-acetate (Table 6), which was also reported by Boeri *et al.* (1955), is attributed to the prevention of metal-catalysed oxidation of the thiol groups.

Marcus & Vennesland (1958) have shown that the reaction catalysed by yeast lactic dehydrogenase does not involve hydrogen atoms attached to the β -carbon atom of lactate. Presumably the lactate molecule is orientated at the enzyme surface in such a way as to permit the transfer of a hydrogen atom from the α -carbon atom to oxidized riboflavin phosphate. The flavin semiquinone so formed would no doubt have a transient existence. However, the probable formation of such an intermediate is supported by the direct spectroscopic observation of an unstable oxidation product of the enzyme, and by the recent demonstration of free radicals formed during oxidations catalysed by several different flavoprotein enzymes (Commoner, Lippincott & Passonneau, 1958).

In fully active preparations of the enzyme both the flavin and haem group appear to be involved in the reduction of ferricyanide, cytochrome *c* and methylene blue. Preliminary determinations of the activation energies (Table 1) suggest that the same

rate-limiting step is involved in the reactions with each of these acceptors. Furthermore, spectrophotometric titration of cytochrome b_2 with lactate (Chance *et al.* 1956) indicates that both the flavin and haem group are reduced simultaneously. However, under certain conditions of inactivation, the rate of reduction of ferricyanide declines more rapidly than does the rate of reduction of the haem group of the enzyme (Table 8); the haem group of inactive cytochrome b_2 may be slowly reduced by active enzyme (Appleby & Morton, 1954; Yamashita *et al.* 1957, 1958). The claim that non-haem iron (Boeri & Tosi, 1956) or other metals (Green & Beinert, 1955) have an essential function in mediating electron transport between the flavin and haem prosthetic group is not substantiated by the results reported here.

Of the other known cytochrome *c*-linked dehydrogenases, succinic dehydrogenase of heart muscle and aldehyde dehydrogenase of pig liver merit comparison with yeast lactic dehydrogenase. Succinic dehydrogenase is associated with cytochrome *b* in heart muscle, but was separated from this by treatment of the lipoprotein complex with butan-1-ol (Morton, 1950, 1955) and shown to be a flavoprotein (Morton, 1955; Kearney & Singer, 1955; Wang, Tsou & Wang, 1956). The purified succinic dehydrogenase contains 4 g.atoms of non-haem iron/mole of enzyme, but this iron does not appear to be involved in electron transfer (Massey, 1958).

Mahler (1956) has stated that liver aldehyde dehydrogenase contains flavin, metal (molybdenum) and haem groups, and thus resembles yeast lactic dehydrogenase. However, the haem content of the aldehyde dehydrogenase is extremely variable in different preparations; in the reduced state, this enzyme shows only very weak visible absorption bands; the difference spectrum is that of a typical flavoprotein (Mahler, Mackler, Green & Bock, 1954). There is as yet no evidence that the haem group is reversibly oxidized and reduced during catalysis by this enzyme.

Yeast lactic dehydrogenase, being a true cytochrome and having both flavin and haem prosthetic groups, thus differs from both succinic and aldehyde dehydrogenases, and from any other dehydrogenase as yet described.

SUMMARY

1. Chemical and enzymic studies with crystalline yeast lactic dehydrogenase are described. The absorption spectrum of the lactate-reduced enzyme shows maxima at 556.5, 528, 424, 330 and 265 $m\mu$; ϵ at 556.5 $m\mu$ is 38.8×10^3 . There is 1 mole each of protohaem and of riboflavin phosphate present as prosthetic groups/80 000 g. of enzyme. No signifi-

cant amount of non-haem iron, copper, cobalt, molybdenum or other related metal is present. There is 1 g.atom of magnesium/80 000 g. of enzyme; this is probably associated with the deoxyribopolynucleotide component of the crystalline material.

2. Oxidation by hydrogen peroxide, and by added Cu^{2+} ions under aerobic conditions, causes dissociation of the flavin prosthetic group and loss of dehydrogenase activity. Ethylenediaminetetraacetate protects against metal-catalysed inactivation. The mechanism of the inactivation process is discussed.

3. The term cytochrome b_2 applies to the native, enzymically active flavohaemoprotein. A haemoprotein product containing no riboflavin phosphate and devoid of enzymic activity may be obtained from cytochrome b_2 .

4. The enzyme catalyses the oxidation of glycolate, L-lactate and α -hydroxybutyrate only. Lactate is oxidized most rapidly.

5. The activation energies for reduction of heart-muscle cytochrome *c*, potassium ferricyanide and methylene blue under specific conditions are 7.2, 7.6 and 8.7 kcal./mole, respectively. Under specified conditions, reduction of heart-muscle cytochrome *c* proceeds by a first-order reaction, and of ferricyanide by a zero-order reaction.

6. The enzymic and chemical properties of the crystalline cytochrome b_2 are compared with those of non-crystalline preparations obtained by other workers.

This investigation was supported financially by the Division of Plant Industry, C.S.I.R.O. This assistance is gratefully acknowledged. We also wish to thank Barrett's Food Co. Ltd., Melbourne, for the generous gift of substantial quantities of dried yeast, and the Defence Standards Laboratory, Commonwealth Department of Supply, Melbourne, for the spectrographic analyses.

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The Composition of Plum Gums

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(Received 1 May 1959)

Gummosis, or the formation of gums, is a common phenomenon in the plant kingdom, and in temperate climates fruit trees of the Rosaceae family, e.g. peach, plum, almond, etc., are particularly susceptible. These gums may appear as exudates on the epidermal surfaces of the various plant organs or as internal pockets ('stone gum') within the tissues. They are composed predominantly of carbohydrate in the form of inorganic salts of complex acidic polysaccharides.

The biosynthetic mechanisms involved in the formation of gum polysaccharides are not understood. It has been variously suggested that gums are the products of micro-organisms (Grieg-Smith,

1904; Butler & Jones, 1949) and that they are produced from pectin (Higgins, 1919) and from hemi-cellulose (Sands & Gary, 1933). Recent histochemical studies by Ceruti & Scurti (1954) have shown that the precursor of cherry gum (*Prunus avium* L.) is probably a galactan. There seems little doubt that the gums originating from *Prunus* species are metabolic products of the plant normally found in the tissues but produced in excess when the plant is stimulated by mechanical or microbiological injury (Hirst, 1949) or by some physiological disorder. The apparent function of gum formation is to seal off surface wounds and to prevent the spread of invading micro-organisms.