proportional to anserine concentration, the equation above still applies (with a different k_1 value), and the differences between colour yields will give the slopes of straight lines representing the relationship of colour yield to the extent of hydrolysis.

In practice it is found that reaction at 100° with 0·2 μmole of anserine or an equivalent hydrolysate is satisfactory when the extent of hydrolysis is greater than 30%. Below 10% hydrolysis, accurate assessment is difficult and the limitations of the standard Schwartz & Engel (1950) procedure, as applied to anserinase assay, become apparent. The concentrations of reactants cannot be increased appreciably as the $k_1(1-x)$ component of the equation above would exceed the level beyond which Beer's law does not apply, and increases in colour yield are small compared with the remaining anserine.

With standard reaction mixtures (0·2 μmole of dipeptide or an equivalent hydrolysate), the sensitivity of the ninhydrin–amino acid reaction at 35° is about half that at 100°, but interference from anserine is reduced to negligible proportions. By keeping the proportion of enzyme reaction mixture to ninhydrin reagent constant, the volume of hydrolysis mixture has been increased from 0·2 to 1·6 ml. for reaction with 8 ml. of reagent at 35° and dilution to 10 ml. This increased the sensitivity some 400% as compared with that of the standard 100° procedure. By the use of cells with longer path-lengths, the sensitivity may be increased further.

SUMMARY

1. Anserine reacts negligibly slowly with ninhydrin reagent at 35°, whereas its component 1-methylhistidine and β-alanine are reactive.

2. This provides a basis for an improved colorimetric procedure for measuring low levels of hydrolysis of anserine.

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The Metabolism of Acetaldehyde in Mammalian Tissues

REACTIONS IN RAT-LIVER SUSPENSIONS UNDER ANAEROBIC CONDITIONS

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Although a large part of the energy metabolism of mammals may be covered through the metabolism of ethanol, the actual pathway followed when this substance is catabolized is only imperfectly known. It is generally accepted that the first product of ethanol oxidation is acetaldehyde. For the subsequent reactions of this substance there are, however, a number of possibilities among which the oxidation to acetate is perhaps the most obvious. This oxidation may proceed in different ways. The presence in the liver of an aldehyde mutase catalysing the dismutation of two molecules of aldehyde to one molecule of alcohol and one of the corresponding acid was assumed for many years. This enzyme system was studied in considerable detail by Dixon & Lutwak-Mann (1937). They showed that diphosphopyridine nucleotide was necessary as a coenzyme. Adler, Euler & Günther (1938) showed that the mutase contains alcohol dehydrogenase, and Reichel & Burkart (1939) found that addition of a flavoprotein changed the dismutation into an oxidation. Racker (1949) prepared a diphosphopyridine nucleotide-requiring aldehyde dehydrogenase from ox liver and pointed out that the mutase activity observed by Dixon & Lutwak-Mann might be caused by a combination of aldehyde dehydrogenase and alcohol dehydrogenase. The presence of a specific mutase beside the two dehydrogenases has, however, not been disproved.

Apart from oxidation of acetaldehyde other possibilities exist. The reaction with pyruvate, resulting in the formation of acetoin with elimination of carbon dioxide, has been demonstrated in brain tissue by Stotz, Westerfeld & Berg (1944) and Berry & Stotz (1954), and has been studied in

liver preparations by Järnefelt (1955). A specific aldolase catalysing the condensation of glyceraldehyde 3-phosphate and acetaldehyde with formation of deoxyribose 5-phosphate was discovered by Racker (1952) both in bacteria (*Escherichia coli*) and in mammalian tissue (notably liver and thymus). Finally, Gilbert (1957) and Karasek & Greenberg (1957) have investigated a reversible condensation reaction between acetaldehyde and glycine leading to threonine or *allo*threonine. The enzyme was demonstrated in mammalian liver. As a physiological route to threonine this reaction is presumably of small importance, as feeding ethanol and glycine to rats failed to obviate the need for threonine (Karasek & Greenberg, 1957).

In an attempt to ascertain which of the possible pathways in acetaldehyde metabolism play a role under physiological conditions we have commenced by studying the reactions which take place in rat-liver suspensions under conditions where the number of potential pathways for acetaldehyde are limited by the absence of molecular oxygen. A preliminary communication of some of this work has appeared (Rasmussen, Fugmann & Lundquist, 1958).

EXPERIMENTAL

The experimental technique consisted essentially of quantitative analyses by sensitive enzymic methods for a number of substances present in samples removed at definite intervals from a tissue suspension to which acetaldehyde had been added.

Tissue preparations. White rats, 3–6 months old, which had been fasted for 20 hr. were decapitated and bled. The liver was removed immediately and chilled on ice. The tissue was weighed and a suspension prepared by disintegration in a 60 ml. Potter–Elvehjem homogenizer at 0° for about 1 min. with 10 vol. of a solution containing KCl (0·09 M), KH_2PO_4 (0·014 M) and Na_2HPO_4 (0·041 M). Nicotinamide (0·02 M final concn.) was added to the salt solution before use to inhibit diphosphopyridine nucleotidase activity. The disintegration may be performed in a smaller volume and the rest of the buffer solution added after homogenization. The suspension was filtered through gauze to remove shreds of connective tissue and kept at 0° until use. Fasted rats were used to ensure low levels of glycogen, which otherwise prevents the preparation of clear deproteinized extracts suitable for optical tests. Diphosphopyridine nucleotide (DPN) was added in most experiments at a concentration of 200–300 μM.

Apparatus

The glass vessel shown in Fig. 1 was used in most experiments. It consists of a cylindrical container (*A*) with two necks furnished with B 10 standard ground-glass sockets and a side arm with a stopcock (*B*). In one socket is placed a tube (*d*) with a stopcock (*C*) provided with double oblique bore connected to a 0·5 ml. pipette (*a*) and a tube (*b*) with side arm. The tapered ends of the pipette and of tube *b* are joined with a thin plastic tube (not shown in the figure). The other socket is connected through a three-way T-type of stopcock (*D*) and a B 7 standard ground-glass joint to a 5 ml. glass syringe (*E*) and a tube (*c*). The syringe is furnished with a stop at 3·0 ml. and with steel springs which ensure rapid emptying. The whole apparatus is fixed on a Perspex frame and can be shaken mechanically at a frequency of 100 times/min. in a water bath generally maintained at 21°. The syringe is situated outside the water bath.

Use of apparatus. All stopcocks and joints were greased with silicone lubricant. Container *A* was rinsed with 10–15 ml. of tissue suspension, which was removed through the syringe and tube *c*. Tissue suspension (up to 60 ml.) was now measured into *A*, cock *B* opened and the mechanical shaking started. Nitrogen was passed in through the side arm of *b* and out through *B* at a rate of 200 ml./min. The nitrogen was passed from the cylinder through a pressure regulator with escape valve and an efficient wash bottle containing an oxygen-absorbing solution (20 ml. of 25% pyrogallol + 100 ml. of 60% KOH + about 100 mg. of β-anthraquinone sulphonate) before it reached the experimental vessel. After 7 min., DPN solution, sometimes together with other substances, was added carefully through the neck holding the pipette. The joint was lifted only sufficiently to allow the insertion of the tip of the Carlsberg constriction pipette used (Levy, 1936; Bessey, Lowry & Brock, 1946). The stream of nitrogen was continued for 7 min., cock *B* closed and two blank samples of suspension were withdrawn by means of the syringe pipette *E*. The syringe was first flushed with 1·5–2·0 ml. of suspension to wash out the dead space of the outlet tubes and the syringe before the samples were taken. The

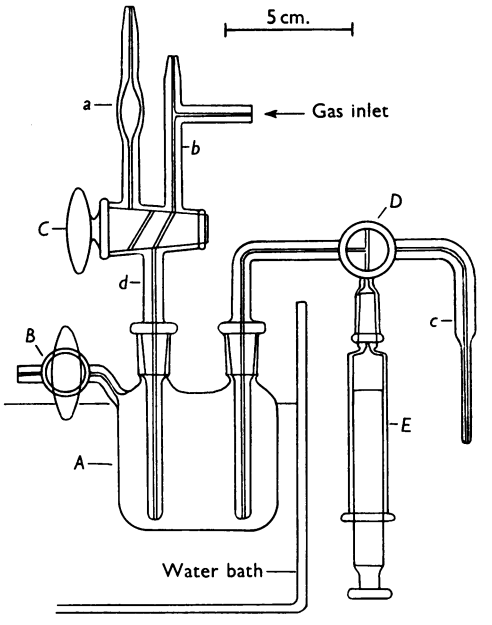

Fig. 1. Apparatus for quick removal of samples of tissue suspension under anaerobic conditions. The apparatus is fixed on a Perspex frame and can be shaken mechanically. Removal of samples through the glass syringe *E* and tube *c* takes 2–4 sec. For further explanation see text.

syringe was then filled (3 ml.) with cock *D* connected to *A*. Then the cock was turned and the contents of the syringe were quickly delivered through the outlet *c* into a glass-stoppered 7 ml. centrifuge tube containing 3 ml. of ice-cold metaphosphoric acid (see Analytical methods). Removal and deproteinization of the sample took 2–4 sec. The blank samples were employed for blank and standard analyses of acetate, ethanol and acetaldehyde.

Cock *B* was opened and the experiment started by addition of acetaldehyde from the pipette *a* by turning cock *C* 180° from the position shown in Fig. 1. In standard experiments 0·5 ml. of 0·02 M-acetaldehyde solution was used. After the addition cock *C* was turned back again and cock *B* closed. The pipette was filled with aldehyde solution before the apparatus was assembled. The filling was performed by attaching a rubber bulb to *a* and sucking the aldehyde solution into the pipette through tube *d*. Cock *C* was turned 180° so that tube *d* was emptied of aldehyde solution before the pipette was connected to the vessel. The tube was flushed with water to remove the aldehyde solution completely.

The first experimental sample was usually taken 30 sec. after the start. The dead space of the outlet channel was flushed with 1·5–2·0 ml. of suspension immediately before each sampling. Before the first sample flushing was performed twice. The timing of the samples depended on the rate of the processes studied. One 3 ml. sample of tissue suspension was sufficient for determination of ethanol, acetate, and acetaldehyde. In experiments where acetate and other analyses were included together with aldehyde and ethanol it was necessary to take two consecutive samples in order to have sufficient material. For determination of DPN and reduced diphosphopyridine nucleotide (DPNH), tissue suspension was collected in tubes filled with nitrogen (see Analytical methods).

Analytical methods

Protein-free extracts. For enzymic determination of acetaldehyde, ethanol, acetate, pyruvate, lactate and α-oxoglutarate deproteinization with metaphosphoric acid was used. Metaphosphoric acid (12 g.) in sticks was dissolved in 100 ml. of water with vigorous mechanical stirring. As this solution hydrolyses rapidly it was kept at 0° and was not used when more than about 4 days old. The metaphosphoric acid-treated samples were centrifuged at 0° for 20 min. (1800 rev./min.).

For acetate analyses the supernatant (2 ml.) was used directly. For determination of aldehyde and ethanol the supernatant was transferred by means of a 1500 μl. Carlsberg constriction pipette to tubes (diam. 15 mm.) which were covered with a piece of Parafilm (Marathon Corp., Menasha, Wis., U.S.A.). The clear solution was neutralized with 0·5 vol. of KOH standardized to give pH 8·1 when added in this amount to the solution. The standardization and appropriate dilution of the stock KOH solution (approx. 2 N) were performed in connexion with each experiment, as the metaphosphoric acid solution changes its titre from day to day. The acid solutions may be kept at 0° for 1–2 days before analysis, whereas neutralized solutions should preferably be analysed on the same day.

For determination of DPN and DPNH, extracts were made by boiling the homogenate without any additions. Long test tubes (200 mm. × 14 mm.) were filled with nitrogen and quickly covered tightly with a piece of Parafilm. The tip of the outlet tube (*c*) from the experimental vessel was allowed to pierce the Parafilm and the 3 ml. sample was placed with occasional shaking in boiling water for 1 min. and then cooled in ice. The coagulated samples were centrifuged at 0° for 15 min. at 15 000 rev./min.

Acetaldehyde. The enzymic method, employing aldehyde dehydrogenase from yeast (Lundquist, 1958), was used. Neutralized extract (2 ml.) was measured into the 1 cm. cuvette of a spectrophotometer (Hilger Uvispek) followed by 1 ml. of the following mixture, which was prepared from the various solutions shortly before use: M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 8·1 (6 vol.); 3 M-KCl (2 vol.); M-ethylenediaminetetra-acetate (EDTA, potassium salt) (1 vol.); 0·1 M-1-mercaptoethanol (1 vol.); 0·015 M-DPN (1 vol.); water (9 vol.).

After stirring, the extinction was measured at 340 mμ, 25 μl. of enzyme (at least 125 units) added and the extinction measured again after 10 min. An aldehyde standard was prepared by addition of 25 μl. of 0·02 M-acetaldehyde soln. to 3 ml. of liver suspension + 3 ml. of metaphosphoric acid. The standard and the blank (suspension before addition of acetaldehyde) were treated exactly as the other samples.

Ethanol. Ethanol was determined essentially by means of modification 3 of Lundquist & Wolthers (1958). Neutralized extract (2 ml.) was mixed with buffer–DPN solution (1 ml.). The buffer solution was prepared as follows. Glycine (30 g.), EDTA (0·6 g.) and semicarbazide hydrochloride (ethanol-free; 5 g.) were dissolved in 200 ml. of water and 2 N-NaOH (50 ml.) was added (pH about 8·8). This solution is stable for several weeks in the refrigerator. Just before use 0·05 vol. of 0·015 M-DPN was added. The extinction at 340 mμ of the mixture of neutralized filtrate and buffer–DPN was measured, yeast alcohol dehydrogenase (0·3 mg. in 25 μl.) was added and the extinction measured after 60–75 min. at room temperature. The enzyme solution was made from the crystal suspension by dilution with 1·5 vol. of water. Standard analyses were performed on a blank sample of liver suspension not containing acetaldehyde; 0·02 M-ethanol soln. (25 μl.) was added to 3 ml. of homogenate after addition of metaphosphoric acid. The standard was treated as the other samples.

Acetate. An enzymic method for determination of acetate was devised for this purpose (Fugmann, Lundquist & Rasmussen, 1958). The method involves two steps. The first is a diffusion in Conway units from the metaphosphoric acid extract (2 ml.) into a small volume of NaOH solution. The second step is similar to the method outlined by Soodak & Lipmann (1948) and Soodak (1957) and involves the acetylation of sulphanilamide by means of a pigeon-liver enzyme preparation.

Pyruvate and lactate. These substances were measured by means of crystalline lactic dehydrogenase from muscle. For pyruvate analyses 2 ml. of neutralized filtrate + 1 ml. of 0·1 M-phosphate buffer (pH 7·4) was employed. The initial DPNH concentration was about 0·1 mM. The change in extinction at 340 mμ which takes place after addition of enzyme (60 μg. in 25 μl.) was measured after 2 min.

For lactate determinations 2 ml. of neutralized extract was mixed with 1 ml. of a solution containing K_2CO_3 (about 1·2 M) and semicarbazide (0·9 M). The pH was adjusted to 10·3 through addition of solid K_2CO_3. Immediately before use DPN (4 mg./ml.) was added to the

solution. The increase in extinction in 1 hr. after addition of lactic dehydrogenase (25 μg.) was measured at 340 mμ.

α-Oxoglutarate. This substance was determined essentially as pyruvate. The same buffer and DPNH concentrations were used. Glutamic dehydrogenase (5 μl.) and half-saturated $(NH_4)_2SO_4$ soln. (45 μl.) were added and the decrease in extinction at 340 mμ was measured after 4 min.

DPN and DPNH. The method of Glock & McLean (1955*a*) was used in some experiments. It has the advantage of sensitivity but requires large amounts of DPNH–cytochrome *c* reductase, which is very time-consuming to prepare, and of cytochrome *c*, which is expensive. In experiments where DPN was added other and simpler methods could be used. The difficulty encountered in these analyses was to prevent reoxidation of reduced DPN in the interval between withdrawal of samples from the experimental vessel and complete destruction of the enzymes involved in DPNH oxidation. A number of methods were tried and finally the following procedure, which is partly based on the method of Spirtes & Eichel (1954), was found to give reproducible results and good recovery of added DPNH.

The samples were removed and coagulated by heating as already described. DPN and DPNH were both determined by means of alcohol dehydrogenase. For DPN determination 1 ml. of the extract was mixed with water (875 μl.), ethanol (100 μl.) and 1 ml. of a buffer solution identical with the glycine-semicarbazide buffer used for the ethanol determination. The extinction at 340 mμ was measured, alcohol dehydrogenase (0·3 mg. in 25 μl.) added and the extinction measured again after 30 min. The increase is a measure of the DPN concentration. For DPNH determination 1 ml. of extract was mixed with water (875 μl.), 2 M-acetaldehyde (100 μl.) and 1 ml. of buffer of the following composition: to 0·1 M-phosphate buffer, pH 7·4 (500 ml.), was added M-EDTA (potassium salt; 25 ml.) and 0·1 M-1-mercaptoethanol (25 ml.). The extinction was measured at 340 mμ, alcohol dehydrogenase solution (0·3 mg. in 25 μl.) was stirred in and the decrease in extinction determined after 30 min. The concentrations were calculated from a molar extinction for DPNH of $6{\cdot}22 \times 10^3$.

Materials. DPN and DPNH were obtained from Boehringer und Söhne, Mannheim (DPN-reinst, DPNH-reinst). Crystalline alcohol dehydrogenase from yeast was either prepared according to Racker (1950) or purchased from Boehringer und Söhne. Crystalline lactic dehydrogenase (from muscle), glutamic dehydrogenase (from liver) and cytochrome *c* were likewise obtained from Boehringer und Söhne. Adenosine triphosphate (cryst. sodium salt), coenzyme A and α-oxoglutarate were obtained from Sigma Chemical Co., oxaloacetate from Fluka (Buchs, St Gallen, Switzerland) and other chemicals were analytical-grade products.

Acetaldehyde was redistilled and diluted with water to approximately 2 M. This solution keeps for at least half a year at 4°. The concentration was occasionally checked by titration. A pyruvic acid stock solution (M) was prepared from pyruvic acid freshly distilled *in vacuo*. All solutions were made up in water passed through a column of Dowex 50 ion-exchange resin in the hydrogen form.

RESULTS

A typical experiment is shown in Fig. 2. The acetaldehyde concentration decreases nearly linearly to very low values. The slope of the curve generally decreases perceptibly at an aldehyde concentration of about 20 μM, but the shape of the curve below this concentration is not accurately known owing to analytical difficulties. The ethanol concentration increases from nearly zero and reaches a maximum when all aldehyde has disappeared. In experiments of longer duration a very slow decrease of the ethanol concentration after this point has been observed regularly (see Discussion).

The aldehyde disappearance minus the ethanol formation is called the 'aldehyde activity', and simply signifies the rate at which aldehyde is removed along pathways other than reduction to ethanol. Acetate determinations show that the whole of the 'aldehyde activity' is due to oxidation to acetate, as the sum of ethanol, acetaldehyde and acetate remains constant throughout the experiment (Fig. 2). In other words condensation reactions play no measurable part under these experimental circumstances. The enzyme system which causes these rapid changes is very stable. Two experiments performed on the same liver preparation with an interval of 1·5 hr., during which

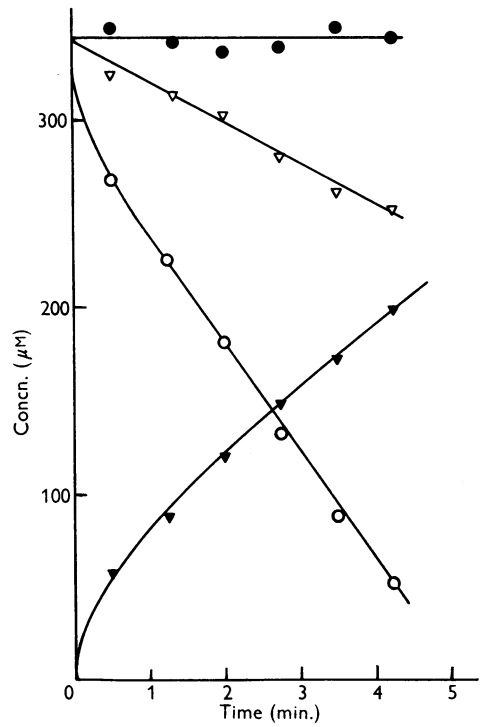

Fig. 2. Standard experiment. Concentration of metabolites as a function of time. ○, Acetaldehyde; ▼, ethanol; ▽, aldehyde + ethanol; ●, sum of acetaldehyde, ethanol and acetate. Initial concentration of DPN was about 200 μM. Temp. 21°.

the preparation had been kept at 0°, gave nearly identical results. This stability makes it feasible to perform many consecutive experiments with the same liver suspension prepared from several (up to 10) rat livers. The acetate formation observed could be caused either through the activity of 'aldehyde mutase' or through Racker's (1949) aldehyde dehydrogenase. If the latter enzyme is responsible it should be possible to dissociate ethanol formation from acetate formation. If no mutase is present the following reactions are believed to account for the aldehyde disappearance:

$$\text{Acetaldehyde} + \text{DPN}^+ \rightarrow \text{Acetate} + \text{DPNH} + \text{H}^+ \quad (1)$$

$$\text{Acetaldehyde} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{Ethanol} + \text{DPN}^+ \quad (2)$$

$$2\ \text{Acetaldehyde} \rightarrow \text{Acetate} + \text{ethanol}$$

If the concentration of DPNH, which together with the acetaldehyde determines the extent of reaction 2, is kept at low level through another enzymic reaction which oxidizes DPNH, it should be possible to limit the formation of ethanol considerably. Among the enzyme systems in liver tissue which might be utilized for this purpose we have considered lactic dehydrogenase, malic dehydrogenase and glutamic dehydrogenase.

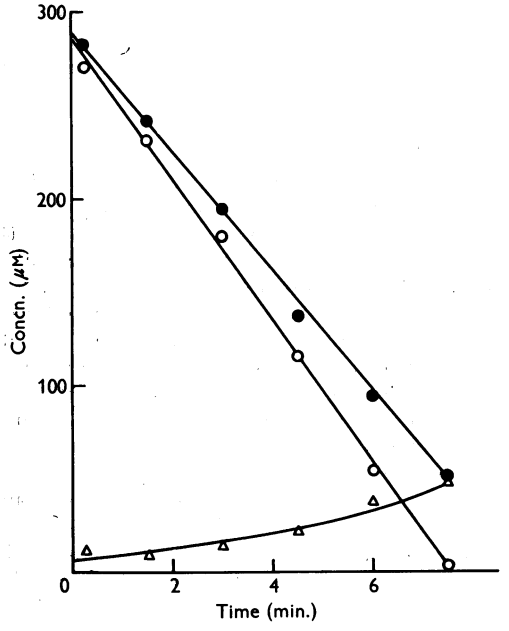

Fig. 3. Acetaldehyde disappearance and ethanol formation in rat-liver suspension in the presence of pyruvate. Initial pyruvate concentration 500 μM. ○, Acetaldehyde; △, ethanol; ●, acetaldehyde + ethanol. Temp. 21°.

Suppression of ethanol formation. When pyruvate is added to liver suspension at a concentration of 0·5 mM the results are as shown in Fig. 3. The rate of ethanol formation is decreased nearly to zero at the beginning of the experiment and increases as the pyruvate is used up. Fig. 4 shows an experiment in which a smaller amount of pyruvate was added. It is evident that the ethanol formation increases when most of the pyruvate has been reduced to lactate. Determination of acetate as well as lactate and pyruvate clearly demonstrates that acetoin is not formed, as all the pyruvate is transformed into lactate and the whole 'aldehyde activity' is accounted for through acetate formation. In 14 experiments where pyruvate was added at a concentration of 500 μM the average ethanol formation was 0·06 μmole/g. of fresh tissue (S.E.M. 0·01).

Similar experiments with addition of oxaloacetate and α-oxoglutarate (Table 1) show the same phenomenon. These results suggest that the 'aldehyde activity' is caused exclusively by aldehyde dehydrogenase.

This conclusion is supported by experiments in which aldehyde dehydrogenase prepared from ox liver according to Racker (1949) was added to rat-liver suspensions. The results are shown in Table 2. The activity of the enzyme added as determined in optical tests was recovered as 'aldehyde activity' in homogenate experiments.

Influence of DPN and acetaldehyde concentration on 'aldehyde activity'. The 'aldehyde activity' is dependent on the initial concentration of DPN in

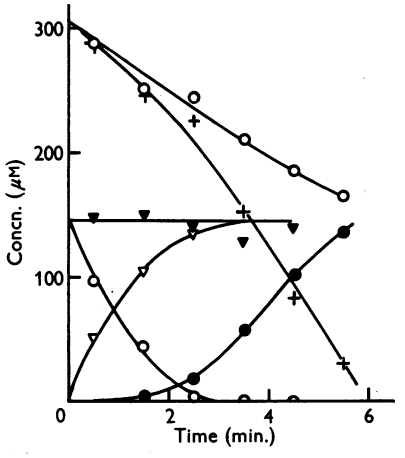

Fig. 4. Disappearance of acetaldehyde and formation of ethanol in rat-liver suspension in the presence of a small amount of pyruvate. ●, Ethanol; +, acetaldehyde; ○ (upper curve), acetaldehyde + ethanol; ○ (lower curve), pyruvate; ▽, lactate; ▼, lactate + pyruvate. Temp. 21°.

Table 1. *Influence of pyruvate, α-oxoglutarate and oxaloacetate on the formation of ethanol from acetaldehyde*

The keto acids were added together with the acetaldehyde at the start of the experiment. Rates are expressed as 10^2 (μmoles/min./g. of fresh liver). Concentrations given for DPN and DPNH are initial values. Temperature, 21°.

Additions	Ethanol formation	'Aldehyde activity'	Keto acid disappearance	DPN (μM)	DPNH (μM)
None	40	22–30	—	150	19
Pyruvate (400 μM)	4	26	42	170	17
α-Oxoglutarate (600 μM) + NH_4Cl (400 μM)	26	28	37	161	14
Oxaloacetate* (600 μM)	6	41	—	137	13

* In this experiment acetaldehyde was determined by means of yeast alcohol dehydrogenase, as the presence of a small amount of malic dehydrogenase in the yeast aldehyde dehydrogenase interfered with the analysis.

Table 2. *Recovery as 'aldehyde activity' of purified liver aldehyde dehydrogenase added to rat-liver suspensions*

The aldehyde dehydrogenase was prepared according to Racker (1949) and dialysed against KCl–phosphate buffer as used for the liver suspension. The enzyme activity was measured by the optical test in the same buffer and with the same concentration of DPN (200 μM) as employed in the homogenate experiments. The activity is expressed as DPNH formed in μmoles/min./l. of final tissue suspension. The experimental vessel contained 45 ml. of liver preparation in all experiments. Temperature 21°.

Additions to the liver suspension	'Aldehyde activity' (μmoles/min./l.)	Ethanol formation (μmoles/min./l.)	Increase in 'aldehyde activity'
None	30	38	—
Pyruvate (500 μM)	31	5	—
KCl–phosphate buffer (15 ml.)	21	38	—
Aldehyde dehydrogenase (15 ml.), corresponding to an activity of 31 μmoles/min./l.	54	34	33
KCl–phosphate buffer (15 ml.) + pyruvate (500 μM)	22	3	—
Aldehyde dehydrogenase (15 ml.), corresponding to 31 μmoles/min./l. + pyruvate (500 μM)	52	0	30

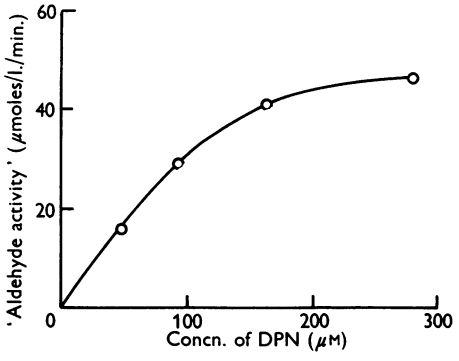

Fig. 5. Influence of concentration of DPN on the 'aldehyde activity'.

the tissue suspension. A number of parallel experiments were performed where the same tissue preparation was employed at different concentrations of DPN. Fig. 5 shows an experiment of this kind. A maximum velocity was approached as the DPN concentration reached 200 μM. A Michaelis constant for the DPN-dependence of 76, 82 and 100 μM was obtained from three experiments of this type. Two similar experiments, but with addition of pyruvate (0·5 mM), gave K_m values within the same range. The K_m for DPN with ox-liver aldehyde dehydrogenase was found by Graham (1951) to be 64 μM at pH 9·3. Thorsteinnson (unpublished) found a value of 43 μM at the same pH. Our results therefore are in agreement with the assumption that the 'aldehyde activity' is caused by aldehyde dehydrogenase, provided that the K_m is independent of pH. Experiments performed without addition of DPN gave aldehyde activities of about one-third of those of the standard experiments. The initial DPN concentration in these cases was about 30 μM (range 26–37 μM). No measurable difference in aldehyde activity was observed when the initial acetaldehyde concentration was varied in the range 0·1–0·6 mM.

Changes in the concentration of DPN and DPNH during acetaldehyde metabolism. In a number of experiments the changes of the DPN and DPNH concentrations during aldehyde disappearance

were measured. Fig. 6 shows a typical experiment. When the tissue suspension has been made anaerobic by a stream of nitrogen for 10 min. the DPNH regularly constitutes 10–20 % of the total coenzyme. On addition of aldehyde the concentration of DPNH falls to about half this value and then rises slowly as the aldehyde is used up. At the end of the experiment the DPNH concentration is very similar to that measured before addition of aldehyde. The sum of DPN and DPNH is seen to decrease steadily throughout the experiment in spite of the considerable concentration of nicotinamide present. When pyruvate was present in the liver suspension the DPNH concentration rapidly fell below the level which could be determined by our methods.

In experiments where DPNH was added initially the level of reduced coenzyme at the moment when aldehyde was added amounted to 55–65 % of the total diphosphopyridine nucleotide concentration. The concentration dropped in less than 30 sec. to the level of about 10 % observed when DPN was added initially. This drop is accompanied by the formation of a corresponding amount of ethanol from acetaldehyde. During the following period, where the aldehyde concentration decreases linearly, a slow rise in DPNH concentration is regularly observed (see Discussion).

Temperature-dependence of the 'aldehyde activity'. Fig. 7 shows an Arrhenius diagram from which an activation energy of 11·5 kcal./mole can be calculated. As all our standard experiments were performed at 21° the results may be converted for 37° simply by multiplication by 2·75.

Magnitude of hydrogenations and dehydrogenations. In 28 experiments performed at 21° where the initial DPN concentration was about 200 μM and the initial acetaldehyde concentration was about 300 μM the mean value ± S.E.M. for the 'aldehyde activity' was 0·23 ± 0·02 μmole/min./g. of fresh tissue, and the ethanol formation was 0·36 ± 0·01 μmole/min./g. of fresh tissue. The results are expressed on the basis of fresh liver weight as this was found to give more uniform results than the use of total protein (quantitative biuret reaction).

It is seen that ethanol formation on the average is considerably higher than the aldehyde activity. In other words we do not always find a simple dismutation. There must be some reaction other than the aldehyde dehydrogenation which furnishes DPNH for reduction of acetaldehyde to ethanol.

The extent of this reaction can readily be calculated from the experimental data. At the end of the experiment the ethanol formed minus the aldehyde activity is a measure of this secondary reaction provided that the final DPNH concentration is identical with the initial DPNH concentration. If the DPNH concentration is higher at the end of the experiment than at the beginning the increase should be added to the difference ethanol formation minus 'aldehyde activity' to obtain the extent of the secondary reaction. Where DPN and DPNH concentrations were measured throughout the experiment a curve may be constructed in this way which shows the time course of the secondary reactions. The velocity of these secondary reactions is in general not constant throughout the experiment, but proceeds faster at the beginning. In

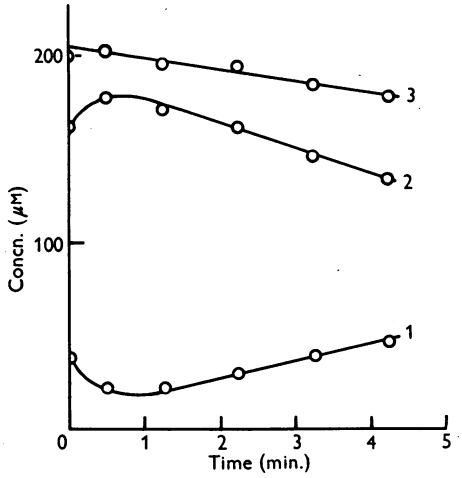

Fig. 6. Changes in DPN and DPNH concentrations during anaerobic acetaldehyde metabolism in a rat-liver suspension. Curve 1, DPNH; curve 2, DPN; curve 3, DPN + DPNH. The initial concentration of acetaldehyde was 280 μM. At 4 min. this had decreased below 15 μM and the concentration of ethanol had reached 160 μM. Temp. 21°.

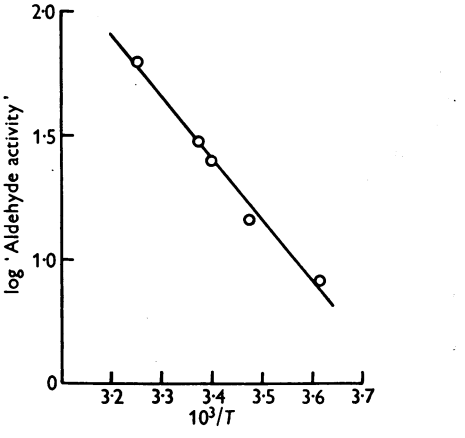

Fig. 7. Temperature-dependence of the 'aldehyde activity'. Initial concentration of DPN 200 μM. Concentration of nicotinamide 0·02 M. T indicates temp. (° K.).

some cases, however, a straight line is obtained. In experiments with excess of pyruvate the DPNH concentration will be so low that variations in the concentration will have no influence stoicheiometrically on the reaction. Here we have simply that the sum of the DPN-reducing reactions must equal the sum of the DPNH-oxidizing reactions. In other words aldehyde dehydrogenase activity (acetate formation) + secondary enzyme activity = alcohol dehydrogenase activity (ethanol formation) + lactic dehydrogenase activity (pyruvate disappearance).

The velocity of the secondary enzyme reactions measured in this way was found to be constant throughout the experiment. The average value for 16 experiments was 0·25 μmole/min./g. of liver ± 0·01 (S.E.M.). In two of these experiments it was possible to compare the magnitude of secondary DPN-reducing reactions with and without addition of pyruvate to the same tissue suspension. It appears (Table 3) that the presence of relatively high concentrations of pyruvate has little effect on the secondary DPN-reducing reactions. In experiments where the DPN concentration was changed it was possible to obtain an impression of the K_m value for DPN of the reaction(s) in question. In two experiments values of 30–50 μM were found. It seems likely that one of the reactions responsible for the secondary DPN reduction is glyceraldehyde phosphate dehydrogenase. The Michaelis constant for this enzyme (from rabbit muscle) is of the same order of magnitude, about 40 μM (Cori, Slein & Cori, 1948).

Acceleration of aldehyde disappearance by pyruvate. Pyruvate has been used in many experiments to reduce the level of DPNH and consequently the ethanol formation. When DPN was added at a concentration of about 200 μM the aldehyde activity was in general increased considerably on addition of pyruvate. In 17 standard experiments at 21° the mean value of the 'aldehyde activity' ± S.E.M. was 0·193 ± 0·01 μmole/min./g. of fresh liver. On addition of pyruvate at a concentration of 500 μM the mean value with the same tissue suspensions was 0·295 ± 0·01. The average increase caused by the pyruvate was 0·102 ± 0·013 μmole/min./g. of fresh liver.

This increase was not due to condensation reactions (acetoin formation), as all aldehyde which disappeared was accounted for as acetate and ethanol, and moreover the sum of lactate and pyruvate was constant throughout the experiment (see Fig. 4).

The activity of aldehyde dehydrogenase from ox liver was independent of the DPNH concentration up to concentrations much higher than those encountered in the tissue suspensions (Fig. 8).

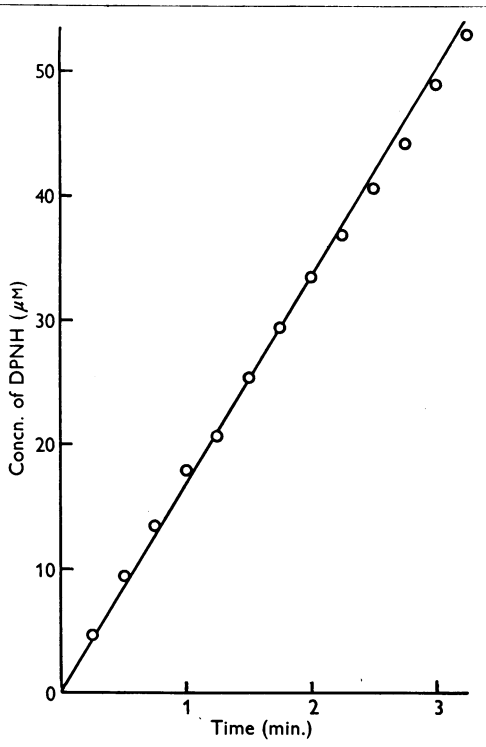

Fig. 8. Reduction of DPN by aldehyde dehydrogenase from ox liver. The initial concentration of DPN was about 200 μM. The enzyme was dialysed against the KCl–phosphate buffer used in experiments with liver suspensions. The DPNH concentration was followed spectrophotometrically at 340 mμ. Temp. 21°.

Table 3. *Net velocity of diphosphopyridine nucleotide-reducing reactions (other than aldehyde dehydrogenation) in liver suspensions*

The secondary reactions were measured both with and without addition of pyruvate. The total diphosphopyridine nucleotide concentration was about 220 μM and the temperature 21° in all experiments. In the experiments without pyruvate the secondary reactions were determined as the difference between ethanol formation and 'aldehyde activity' corrected for changes in the DPNH concentration. All results are given as 10^2 (μmoles/min./g. of fresh liver).

Additions	'Aldehyde activity'	Ethanol formation	Secondary reactions	Pyruvate disappearance
None	21	40	29	—
Pyruvate (500 μM)	23	4	35	54
None	17	42	26	—
Pyruvate (500 μM)	27	5	32	54

DISCUSSION

Under the conditions chosen in this study it is apparent that condensation reactions like those mentioned in the introduction play no measurable part in the removal of acetaldehyde. The aldehyde concentrations used by us are low in comparison with those employed in the studies of Stotz *et al.* (1944), Järnefelt (1955), Karasek & Greenberg (1957), Gilbert (1957) and Racker (1952). This fact could easily account for the failure to observe any of the reactions described by these authors, as the respective enzymes apparently all have rather large Michaelis constants with respect to acetaldehyde. As the acetaldehyde concentrations observed in living mammalian organisms during ethanol metabolism (Wagner, 1957; Lundquist & Wolthers, 1958) are still lower than those employed in the present experiments *in vitro*, we may safely assume that in the normal human organism the various condensation reactions of acetaldehyde are of no quantitative importance for the removal of this substance during metabolism of ethanol. This, of course, does not exclude the possibility that these reactions may take place to a small extent. Lubin & Westerfeld (1945) and Westerfeld (1949) found no support for the belief that acetoin formation during acetaldehyde metabolism is of any quantitative significance.

The concentration in tissue suspensions of DPN, which should be chosen to approximate most closely the conditions in the intact tissue, is difficult to decide. Glock & McLean (1955*b*), Jacobson & Kaplan (1957) and others have found the total concentration of diphosphopyridine nucleotides in rat liver to be 500–600 μg./g. of fresh tissue. A very sizeable part of this is undoubtedly bound to proteins. In order to imitate in tissue suspensions the conditions in the intact tissue it is necessary to have the DPN concentration equal to the concentration of free coenzyme in the fresh liver, which is not known with certainty. The value chosen by us (about 200 μM) is probably not far from the actual concentration of free coenzyme in liver tissue, and the aldehyde activity did not increase appreciably when the DPN concentration was raised above this.

The changes which take place in the ratio of DPN to DPNH with decreasing aldehyde concentration are in agreement with the fact that alcohol dehydrogenase has a lower affinity for aldehyde (K_m 110 μM: Theorell, Nygaard & Bonnichsen, 1955) than has aldehyde dehydrogenase (K_m presumably less than 10 μM, Racker, 1949; see below). The rate of aldehyde dehydrogenation relative to aldehyde reduction to ethanol will increase as the aldehyde concentration decreases, and as a consequence the DPNH concentration will increase. The rate of ethanol formation observed in the beginning of those experiments where DPNH was added is very high in comparison with the aldehyde activity and is indeed compatible with the kinetic constants observed for crystalline liver alcohol dehydrogenase by Theorell *et al.* (1955). As alcohol dehydrogenase therefore is present in our preparations in excess, the aldehyde activity is determined by the amount of aldehyde dehydrogenase, as is also clearly evident from experiments in which purified liver aldehyde dehydrogenase was added (Table 2). In these experiments the ethanol formation was not increased. This may mean that the DPNH concentration, which presumably determines the rate of ethanol formation, is controlled to a large extent by other reactions in which DPNH is formed (see Table 3).

During the major part of the reaction the possibility might be considered that the concentration of ethanol, acetaldehyde, DPN and DPNH approximate to an equilibrium as suggested by Holzer, Schultz & Lynen (1956) for yeast cells. Such a proposition is, however, not tenable in this case, as may be seen from a simple calculation. The ratio DPN/DPNH does not change very much during the experimental period, whereas the ratio aldehyde/ethanol changes by a factor of more than 100. As the pH is constant the equilibrium conditions cannot be fulfilled. In all experiments where samples were removed for some time after all aldehyde had disappeared, it was noted that the ethanol concentration reached a maximum near the point where no measurable aldehyde was left. The decrease after the maximum is very slow (about 4 μM/min.). The ethanol concentration is determined by the two processes catalysed by alcohol dehydrogenase:

$$\text{Ethanol} + \text{DPN}^+ \underset{2}{\overset{1}{\rightleftharpoons}} \text{Acetaldehyde} + \text{DPNH} + \text{H}^+.$$

At the maximum point of the ethanol curve the two processes must proceed at the same rate, in other words here an equilibrium is established between acetaldehyde, ethanol, DPN and DPNH. From the known values of the concentration of H^+ ions, DPN, DPNH and the equilibrium constant determined by Racker (1950), the ratio of acetaldehyde concentration to ethanol concentration at the maximum point is calculated to about 2×10^{-3}. A value of 200 μM for the ethanol concentration is frequently observed. This should give an acetaldehyde concentration of 0·4 μM. At this low concentration the aldehyde dehydrogenation must proceed very slowly and will determine the decrease in ethanol concentration after the maximum. From the decrease in ethanol it should therefore be possible to determine the K_m for aldehyde dehydrogenase, as the maximum velocity of the

reaction ('aldehyde activity') is known. A value of 0·2–0·5 μM is suggested by our measurements, but experiments designed to determine this constant have not been performed so far.

The ratio DPNH/DPN observed under anaerobic aldehyde metabolism and still more in the experiments where pyruvate was added is very low compared with the measurements of diphosphopyridine nucleotides in fresh liver, where the value of this ratio of about 0·3 has been reported (Glock & McLean, 1955*b*). In the untreated tissue a very large proportion of the reduced coenzyme is, however, bound to proteins, so the ratio of the free DPNH to free DPN may be much lower than that measured for total coenzyme. Thus Holzer *et al.* (1956) estimate the ratio of free DPNH/DPN in anaerobic yeast cells to 1/400, whereas the direct measurement of total DPNH/DPN (Holzer, Goldschmidt, Lamprecht & Helmreich, 1954) gave a value of 1/3.

Effect of keto acids on the 'aldehyde activity'. An effect of pyruvate and other keto acids similar to our finding in liver suspensions has been reported by Green, Needham & Dewan (1937). They studied a number of dismutation reactions, including the dismutation of acetaldehyde in crude soluble extracts of skeletal muscle and liver. In some cases a large increase in acid formation was observed on addition of keto acids such as oxaloacetate and pyruvate. In many cases this effect was undoubtedly caused by the increased DPN concentration furnished by reduction of the keto acid. With acetaldehyde this explanation can hardly account for the observations. In our experiments the increase in DPN concentration on addition of pyruvate cannot exceed about 10%, which at the concentration of DPN employed will have only negligible effect on the rate of aldehyde dehydrogenation. The possibility that DPNH has an inhibitory effect on the process and that this inhibition is released when the DPNH is oxidized by pyruvate seems also to be ruled out, as no inhibition by DPNH could be observed with purified aldehyde dehydrogenase (Fig. 8).

Nygaard & Rutter (1956) and Astrachan, Colowick & Kaplan (1957) observed that DPNH bound to glyceraldehyde phosphate dehydrogenase could react with some other dehydrogenases without dissociation to free DPNH. In some cases the reaction rate was even greater with bound DPNH than with the free coenzyme. This observation could possibly explain the effect of pyruvate on the 'acetaldehyde activity'. If a complex between lactic dehydrogenase (LDH), DPNH and aldehyde dehydrogenase (ALDH) were formed, the following reactions could be envisaged:

LDH–DPNH–ALDH + pyruvate →
LDH–DPN–ALDH + lactate.

The LDH–DPN–ALDH complex may either react directly with acetaldehyde:

LDH–DPN–ALDH + acetaldehyde →
LDH–DPNH–ALDH + acetate,

or it may dissociate before the reaction of acetaldehyde with ALDH–DPN. Since presumably the dissociation of DPNH from ALDH is the rate-limiting step in the aldehyde dehydrogenation, as with liver alcohol dehydrogenase (Theorell & Chance, 1951), the formation of the complex with lactic dehydrogenase and the subsequent reduction without dissociation would permit a circumvention of the rate-limiting step and therefore an acceleration of the 'aldehyde activity'.

The important discovery by Vennesland and coworkers of stereospecificity of enzymes with regard to the binding of the DPN molecule is of interest in this connexion. It would seem probable that the condition for the sharing of one DPN molecule by two different enzyme molecules would be that the enzymes display opposite stereospecificity. Levy & Vennesland (1957) have found aldehyde dehydrogenase from liver to have α-specificity. The same specificity was found for lactic dehydrogenase and malic dehydrogenase, which therefore should not be capable of reacting with aldehyde dehydrogenase. The lactic and malic dehydrogenases were, however, obtained from muscle tissue. The stereospecificity of the corresponding enzymes from liver has not been determined. We cannot therefore at present decide whether our results are in agreement with the assumption that opposite stereospecificity is a condition for the sharing of diphosphopyridine nucleotides by dehydrogenases.

SUMMARY

1. A technique has been devised which permits the rapid (2–4 sec.) and accurate removal and deproteinization of samples of tissue suspensions under anaerobic conditions.

2. Under anaerobic conditions acetaldehyde is transformed rapidly into ethanol and acetate in suspensions of rat liver in a potassium chloride–phosphate solution. Addition of diphosphopyridine nucleotide accelerates the processes. That part of the aldehyde disappearance which is not caused by ethanol formation (the 'aldehyde activity') approaches a maximum value at a diphosphopyridine nucleotide concentration of about 200 μM.

3. The 'aldehyde activity' is independent of the acetaldehyde concentration in the range examined (0·1–0·6 mM).

4. The whole of the aldehyde removal is accounted for by formation of ethanol and acetate. Condensation reactions such as acetoin synthesis did not take place at the low aldehyde concentrations employed. It is concluded that during

ethanol metabolism in the living organism condensation reactions of acetaldehyde can be of no quantitative importance.

5. Addition of pyruvate or other keto acids reduced the ethanol formation to a negligible value. It is therefore concluded that rat liver does not contain any true aldehyde mutase.

6. The ethanol formation is frequently larger than the aldehyde dehydrogenation. Evidence is obtained that this is due to reduced diphosphopyridine nucleotide, formed at a considerable rate from substrates present in the liver.

7. The concentration of diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide during aldehyde metabolism was followed and the significance of the results is discussed.

8. Addition of pyruvate increased the rate of aldehyde dehydrogenation by about 50%. It is suggested that this result may be caused by the formation of a complex between aldehyde dehydrogenase, lactic dehydrogenase and reduced diphosphopyridine nucleotide, which could be oxidized by pyruvate. The dissociation of reduced diphosphopyridine nucleotide from the aldehyde dehydrogenase, which is presumably the rate-limiting step, would in this way be circumvented.

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A Ribonucleoprotein Component of *Escherichia coli*

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Although aqueous solutions prepared from disrupted bacteria contain macromolecules of many different kinds, a simple pattern, which is common to the several species so far studied, is observed when such extracts are examined in the analytical ultracentrifuge. This is due chiefly to the abundance and ubiquity of a component of the cytoplasm that sediments at about 40 Svedberg units (40s); our present studies suggest that smaller peaks observed for boundaries of 29s and 20s may be due to particles formed by breakdown of the '40s component'. Schachman, Pardee & Stanier (1952) used five methods for disrupting cells of six different species of bacteria and showed that most of the ribonucleoprotein of extracts sedimented with their 40s components, which, in the electron