the *Neurospora* or bacterial mutants. In addition, it seems improbable on structural grounds that these benzenoid compounds would be intermediates in the synthesis of such hydroaromatic compounds as prephenic acid. It is not known at present whether vanillic acid is formed by methylation of the 3-hydroxyl group of dehydroshikimic acid followed by dehydration, or by direct methylation of protocatechuic acid. Gross *et al.* (1956) have shown that vanillic acid is a potent inducer of protocatechuic acid oxidase in *Neurospora*, and therefore it is of added interest that this compound occurs naturally in *Neurospora*.

SUMMARY

1. Some strains of *Neurospora* showing a nutritional requirement for aromatic compounds have been obtained. Preparation of multiple mutants is described.

2. Evidence for the presence of protocatechuic acid, vanillic acid and dehydroshikimic acid in culture filtrates of some of these mutants is presented.

3. The possibility of a central role for dehydroshikimic acid in the synthesis of aromatic compounds by *Neurospora* is discussed.

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Enzymic Determination of Acetaldehyde in Blood

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The determination of acetaldehyde in blood is of interest in the study of ethanol metabolism, where this substance may be expected to occur as an intermediate, especially during treatment with substances such as tetraethyl thiuram disulphide, [bis(diethylthiocarbamoyl) disulphide], which presumably interfere with aldehyde breakdown. The methods available are, however, not entirely satisfactory. Among the purely chemical methods the most reliable is undoubtedly that of Stotz (1943) based on the colour reaction with *p*-hydroxydiphenyl in strong sulphuric acid. The procedure requires, however, distillation involving special apparatus, and is very sensitive to trace contamination. Moreover, interference by various normal metabolic products cannot be excluded.

The elegant method of Burrige, Hine & Schick (1950) involves the use of Conway micro-diffusion units. Acetaldehyde is caught in semicarbazide in a buffer solution and the light absorption of the semicarbazone at 224 m μ is used for the estimation. Unfortunately this method, though very sensitive, is not sufficiently specific. Acetoacetate in the blood will give rise to acetone under the analytical conditions, and this substance interferes in the measurement (see below).

Enzymic methods for determination of acetaldehyde have also been suggested (Holzer, Holzer & Schultz, 1955; Racker, 1957). The oxidation of reduced diphosphopyridine nucleotide (DPN) by aldehydes, catalysed by yeast alcohol dehydrogenase, may be used quantitatively, although the

method is not very accurate at low levels of aldehyde because it involves the measurement of a small decrease in a rather large optical density. Moreover, the reaction with aldehyde is not quantitative when large amounts of alcohol are present, because the equilibrium is changed towards formation of aldehyde. This method has been employed by Brahm-Vogelsanger & Wagner (1957).

Aldehyde dehydrogenase from ox liver (Racker, 1949) would appear to be a very suitable enzyme because the equilibrium is totally in favour of acetate and the enzyme has a very small Michaelis constant towards aldehyde. The reduction of DPN, even by extremely small amounts of aldehyde, consequently proceeds stoichiometrically within a short time. There are, however, certain disadvantages in this method, which we have tried extensively for the determination of acetaldehyde in liver homogenates. The first is that the enzyme is rather difficult to prepare and not very stable. The second and major disadvantage is the presence of lactic and malic dehydrogenases in the enzyme preparations made according to Racker (1949). Several attempts to remove these enzymes, which interfere badly in the analysis of tissue extracts containing lactate and other intermediary metabolites, were unsuccessful and consequently we looked for another source of aldehyde dehydrogenase. Black (1951) has purified a potassium-requiring enzyme from baker's yeast, which catalyses the dehydrogenation of acetaldehyde to acetic acid with either DPN or triphosphopyridine nucleotide (TPN) as coenzyme. This enzyme is very easy to prepare in a state of sufficient purity. The preparations contain no DPN-requiring lactic dehydrogenase or other interfering enzymes and are stable for several months at -20° .

The kinetic properties of the enzyme are very different from those of aldehyde dehydrogenase from liver. The affinity between aldehyde and enzyme is very small, and even though the equilibrium is situated far towards acetate, acetaldehyde is not quantitatively oxidized to acetate. The rate of the reaction is, however, proportional to the aldehyde concentration within rather wide limits and is employed for the quantitative determination of aldehydes. The specificity of Black's aldehyde dehydrogenase is similar to that of alcohol dehydrogenase and liver aldehyde dehydrogenase and is thus far from ideal, as a number of aliphatic and even aromatic aldehydes are attacked. For the study of alcohol-acetaldehyde metabolism this lack of strict specificity is, however, of no importance.

EXPERIMENTAL

Procedure for aldehyde determination in blood plasma by the diffusion technique. As the mixing of reagents and blood plasma in the Conway units as performed by Burridge *et al.*

(1950) proved difficult, the procedure was slightly modified. Plasma, 0.67 N- H_2SO_4 and 10% (w/v) sodium tungstate are mixed thoroughly in glass-stoppered centrifuge tubes in the cold and 5 ml. of the mixture is transferred to the Conway unit. The rest of the procedure is in accordance with Burridge *et al.*

Preparation of yeast aldehyde dehydrogenase. Our procedure differs in some respects from that of Black (1951, 1955). The starting material is commercial dry yeast (Danisco). This yields much less enzyme than fresh baker's yeast, but does not require the use of liquid air. Dry yeast (100 g.) is extracted at room temperature with 500 ml. of 0.3 M- K_2HPO_4 with mechanical stirring for 2 hr., while the pH is kept at 8.6 by addition of conc. aq. NH_3 soln. The suspension is kept at 4° overnight and centrifuged at 2000 g for 25 min. The nearly clear supernatant is treated as described by Black (1951, 1955). Before collection of the precipitate, formed in step 3 by addition of acid to pH 5, the acidified solution is kept for 2 hr. at 4° . The precipitate is dissolved as described by Black, and the solution is reacidified to pH 5. The precipitate is collected by centrifuging and dissolved in approx. 10 ml. of buffer containing 0.025 M- K_2HPO_4 and 0.001 M-cysteine hydrochloride. The pH is adjusted to 6.3 and traces of turbidity are removed by centrifuging. This reprecipitation is performed to remove traces of alcohol dehydrogenase. The enzymic activity of the final solution is measured according to Black. It is regularly found to contain about 200 000 units of enzyme. Alcohol dehydrogenase is measured according to Racker (1950), except that the buffer contains 0.07 M-semicarbazide. The optical density at 340μ is followed for 10 min. The increase should be less than 0.01 for 25 μ l. of enzyme. The enzyme preparation is distributed in small tubes containing approx. 10 000 units each and kept at -20° . It keeps well for several months under these conditions.

Determination of acetaldehyde in blood plasma

Metaphosphoric acid (0.75 M). Sticks (12 g.) of metaphosphoric acid (Merck) are powdered in a mortar and dissolved in 100 ml. of water. This product contains a considerable amount of $\text{Na}_2\text{P}_2\text{O}_7$, added to make the product hard. Pyrophosphate does not interfere unfavourably in the analyses. The solution is kept well-stoppered in the refrigerator, but even at 0° a fairly rapid hydrolysis takes place. The solution is titrated before use and should be discarded when more than a week old.

Potassium hydroxide (1.25 M). A volume (0.5 ml.) of this solution is used to neutralize 2 ml. of deproteinized blood plasma. If the metaphosphoric acid solution is partially hydrolysed it is convenient to dilute a 1.5 N-KOH stock solution according to the titre of the metaphosphoric acid so that 0.5 ml. will neutralize 2 ml. of plasma filtrate.

DPN-buffer mixture. This is prepared by mixing M-KCl (3 vol.), M-2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer, pH 8.0, (6 vol.), 0.1 M-ethylenediaminetetraacetate (0.5 vol.), 0.1 M-mercaptoethanol (0.5 vol.) and 0.015 M-DPN (0.5 vol.).

Stock solution of acetaldehyde (2 M). Freshly distilled acetaldehyde is diluted with approx. 9 vol. of water. The concentration is determined by titration. This solution keeps well in the refrigerator, but should be checked by titration every second month.

All chemicals used were of analytical grade. DPN was the product of C. F. Boehringer und Soehne.

Deproteinization. Blood is collected in plastic centrifuge tubes, which are immediately placed in ice and closed with plastic stoppers. The blood is centrifuged at 4° at 2000 *g* for 10 min. No clotting is observed under these conditions. Plasma (2 ml.) is measured into glass-stoppered, 8 ml. centrifuge tubes containing 2 ml. of ice-cold 0.75 *M*-metaphosphoric acid. After thorough mixing and centrifuging, 2 ml. of the clear supernatant is transferred (by a semi-automatic Carlsberg pipette) to cold, stoppered tubes and neutralized by 0.5 ml. of KOH solution (1.25 *M*). At this stage the samples may be left in the refrigerator overnight without significant loss of acetaldehyde.

Measurement. The neutralized filtrate (2 ml.) is mixed with 1 ml. of the DPN-buffer mixture and placed in 3 ml. cuvettes in a Hilger Uvispek spectrophotometer (light path 1 cm.). The optical density at 340 *mμ* is measured and 25 *μ*l. or a volume containing at least 250 units of yeast aldehyde dehydrogenase is added with simultaneous stirring. The optical density is measured again after the lapse of 10 min. A polyethylene lid on the cuvettes prevents evaporation of aldehyde during the measurement. For each series of analyses blank and standard solutions are included. The blank analysis is made with metaphosphoric acid diluted 1:1 and neutralized with KOH. The standard is made by addition to this solution of 1–10 *μ*g. of acetaldehyde (25 *μ*l. of a suitably diluted stock solution). The increase in optical density in 10 min. of the blank analysis is in general around 0.01, provided that the reagents contain no trace of aldehyde. This value is subtracted from all other readings and the concentration of aldehyde calculated from the proportionality between concentration and increase in optical density (see Fig. 1). It is important to keep the temperature below 15° to limit evaporation of aldehyde.

RESULTS

Diffusion method. In a series of ten analyses of the same plasma with acetaldehyde added to a concentration of 9 *μ*g./ml., the optical density ($\pm$ S.D.) at 224 *mμ* was 0.334 ± 0.011 . Acetone added to blood plasma gave an optical density of 0.12 for 10 *μ*g. of acetone/ml. of plasma. Acetone itself may not normally be present in blood to any measurable extent, but the possibility that aceto-

acetate under the conditions of the analysis may give rise to acetone was examined. A sodium acetoacetate solution, prepared by alkaline hydrolysis of the ethyl ester (Seeley, 1955), was concentrated *in vacuo* at low temperature to remove traces of free acetone. Under the conditions used for analysis of plasma it was found that 2–5 % of the acetoacetate was transformed into acetone, thus giving rise to a significant error. A concentration of acetoacetate in blood plasma about 10 mg./100 ml. will result in an optical density corresponding to about 1 *μ*g. of acetaldehyde/ml. The magnitude of this error in analyses of blood from a normal individual is illustrated in Table 1.

Optimum conditions for the enzymic determination of acetaldehyde

Deproteinization. A number of different methods for deproteinization have been tried. Trichloroacetic acid inhibited the yeast enzyme by about 50 %, and perchloric acid interfered seriously with the determination. Analysis of blood plasma could be performed after zinc or cadmium deproteinization, but very slight deviations from the correct pH after addition of the reagents (zinc sulphate and potassium hydroxide) led to low recoveries or inhibition of the enzyme. Metaphosphoric acid was found, however, to be applicable for deproteinization both of blood plasma and tissue homogenates.

Activity of the enzyme. The rate of the reaction between DPN and acetaldehyde is proportional to the concentration of aldehyde up to 0.1 *M*. It is convenient to use an interval of 10 min. for the measurement. This gives an increase in absorption corresponding to approximately half conversion into acetate. When the increase in optical density

Table 1. Comparison of diffusion method and enzymic method for the determination of acetaldehyde in blood plasma

Four blood samples were taken from a subject fasted for 12 hr., at intervals of approx. 1 hr. All analyses were made in duplicate.

Sample no.	Acetaldehyde in plasma (<i>μ</i> g./ml.)	
	Diffusion method	Enzymic method
1	0.9	0.1
2	1.0	0.1
3	1.0	0.1
4	1.0	0.1

Fig. 1. Relation between concentration of acetaldehyde (*μ*g. of acetaldehyde in the cuvette) and the increase in optical density at 340 *mμ* in 10 min. The aldehyde was dissolved in 2 ml. of neutralized metaphosphoric acid + 1 ml. of DPN-buffer mixture and 25 *μ*l. of enzyme was added. The points represent average values of two determinations.

Table 2. *Reaction of different aldehydes with yeast aldehyde dehydrogenase*

Aldehyde solution (25 μ l.) was added to 3 ml. of neutralized metaphosphoric acid + DPN-buffer mixture. The aldehydes, except trichloroacetaldehyde and glyceraldehyde, were freshly distilled and showed the correct boiling point.

	Concn. (mm)	Increase in optical density at 340 m μ	Percentage of the value for acetaldehyde*
Acetaldehyde	5.3	0.148	100
Formaldehyde	5.0	0	0
Propionaldehyde	4.85	0.151	111
n-Butyraldehyde	4.83	0.107	79
iso-Butyraldehyde	4.86	0.056	41
Crotonaldehyde	5.34	0.160	107
Trichloroacetaldehyde	4.90	0	0
D,L-Glyceraldehyde	4.80	0	0
Benzaldehyde	5.28	0.179	121
Salicylaldehyde	5.20	0	0
Phenylacetaldehyde	5	0.033	24

* The optical densities were corrected to the same concentration.

Fig. 2. Influence of the concentration of yeast aldehyde dehydrogenase on the reaction rate expressed as increase in optical density at 340 m μ in 10 min. The reaction mixture contained 7.4 μ g. of acetaldehyde in 2 ml. of neutralized metaphosphoric acid + 1 ml. of DPN-buffer mixture. The measurements were corrected for variation in blank value caused by variations in the amount of enzyme preparation added. The points are average values of two determinations.

Table 3. *Recovery of acetaldehyde added to blood plasma*

Acetaldehyde added (μ g./ml.)	Acetaldehyde found (μ g./ml.)	Recovery (%)
0	0.2	—
2.32	2.52	100
2.32	2.52	100
2.32	2.70	108
4.62	4.73	98
4.62	4.73	98
4.62	4.66	97

the enzyme is more stable at or below pH 8 we have found this to give better results.

Specificity of the method. A number of aldehydes react with yeast aldehyde dehydrogenase under the conditions used for analysis of blood and tissue. Table 2 presents a summary of the results.

Recovery of acetaldehyde added to blood. It is important to keep blood samples cold and well-stoppered to prevent loss of acetaldehyde by evaporation. Separation of plasma should be done as soon as possible, as aldehyde seems to disappear more rapidly from whole blood than from plasma (Stotz, 1943). Acetaldehyde added to plasma or serum was quantitatively recovered when deproteinization was performed with metaphosphoric acid, and at a level of acetaldehyde as low as 1 μ g./ml. Table 3 shows the recovery of acetaldehyde added to blood plasma.

Accuracy and sensitivity. An impression of the accuracy of the method was obtained through independent analyses of one plasma sample. For each analysis 2 ml. of the plasma was precipitated with metaphosphoric acid. For eleven samples containing 4.6 μ g./ml. of acetaldehyde the increase in optical density $\pm$ s.d. in 10 min. was 0.101 ± 0.002 . The sensitivity of the method is dependent on the stability of the spectrophotometer employed. We

under our analytical conditions is plotted against concentration of aldehyde, proportionality is found within a quite satisfactory range (see Fig. 1).

When the concentration of enzyme is increased above a certain level the reaction rate becomes approximately constant (Fig. 2). We find it convenient to employ 250 units for each measurement. In this way reproducible results are found and the influence of small amounts of inhibitory substances is eliminated.

Hydrogen-ion concentration. A sufficient amount of tris buffer is used to ensure that the pH is near 8 in the final reaction mixture, if the metaphosphoric acid filtrates have been neutralized as described in the Experimental section. The reaction proceeds somewhat faster at more alkaline reaction, but as

have consistently been able to detect an increase in optical density of 0.002 above the reagent blank. This corresponds to a limit for the detection of acetaldehyde in plasma of approx. 0.1 $\mu\text{g./ml.}$

DISCUSSION

As seen in Table 2 the method will measure a number of aldehydes besides acetaldehyde. None of these is, however, believed to occur in animals. It should nevertheless be kept in mind that the concentrations found by the enzymic method represent maximum values, even though they are considerably lower (Lundquist, F. & Wolthers, H. unpublished work) than those observed by the chemical methods (Raby, 1954).

The presence of enzymes other than aldehyde dehydrogenase in the preparation used may result in errors. Alcohol dehydrogenase would cause too high results when alcohol is present. As estimation of aldehyde is of interest primarily in connexion with alcohol metabolism, it is, of course, important to avoid this error. With the procedure described for the purification of the enzyme, alcohol dehydrogenase is completely removed. Numerous experiments have been performed with high concentration of ethanol in biological material and the absence of interference in the analytical method has been established. In order to demonstrate this lack of interference it proved necessary to purify the ethanol by distillation over metal sodium to remove traces of aldehyde. Ethanol thus purified and added to blood plasma in amounts from 0.1 to 2% had no influence on the measurement. Addition of acetate to plasma up to 0.3M is likewise without any influence on the measurement of acetaldehyde.

Other DPN-dependent enzymes may contaminate the preparation employed and could attack substrates present in deproteinized filtrates from biological material. This possibility cannot be excluded completely, but the virtual absence of increase in optical density on examination of liver homogenates, which presumably contain a number of intermediary metabolites, shows that this source of error is of no practical importance.

The concentration of acetaldehyde measured in blood plasma from normal subjects during metabolism of ethanol is extremely low (about 0.2 $\mu\text{g./ml.}$, Lundquist, F. & Wolthers, H. unpublished work), and near the limit of detection for the present method. It would therefore be most desirable to be able to increase the sensitivity. Various possibilities have been considered without much success so far. Theorell, Nygaard & Bonnichsen (1955) have employed the fluorescence of reduced DPN to determine very small concentrations of this substance. Preliminary experiments showed, however, that

the fluorescence of protein-free filtrates from blood and tissues is so large that the increased sensitivity of the measurement of reduced DPN is completely compromised.

Another possibility is to measure the DPN formed from reduced DPN when alcohol dehydrogenase is used. DPN, under the conditions described by Robinson, Levitas, Rosen & Perlzweig (1947) and Carpenter & Kodicek (1950), gives a fairly strong fluorescence which may be used for quantitative measurement. Preliminary experiments made with this method were, however, disappointing. It appeared that the presence of a large excess of reduced DPN inhibits the reaction so that the overall sensitivity is not better than that of the aldehyde dehydrogenase method.

If the fluorescence method were combined with a distillation of the material to be analysed, it should be possible to increase the sensitivity some ten times, but so far no experiments in this direction have been performed.

SUMMARY

1. The determination of acetaldehyde in blood by diffusion into semicarbazide is subject to interference from acetoacetate, which is partly decarboxylated to acetone during the procedure.

2. A method is described based on the use of a potassium-requiring aldehyde dehydrogenase easily prepared from commercial dry yeast. The rate of formation of reduced diphosphopyridine nucleotide under standard conditions is proportional to the aldehyde concentration within a considerable range.

3. Under the analytical conditions chosen a number of aldehydes will react; the specificity is therefore not complete.

4. The accuracy of the method at very low aldehyde concentrations is better than that obtainable with other methods. The limit for detection of acetaldehyde in biological material is around 0.1 $\mu\text{g./g.}$

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The Colorimetric Determination of Dipeptides by means of Oxygenated Cobalt Complexes

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Numerous methods have been proposed for the determination of dipeptide hydrolysis (Martin & Synge, 1945), but no single one of them satisfies the dual requirements of rapidity and accuracy. Procedures based on acid-base titration lack precision because of the large blank due to the substrate; this is true also of the colorimetric-ninhydrin (Schwartz & Engel, 1950) and ammonia-ninhydrin (MacFadyen, 1944) methods. Measurement of the carbon dioxide evolved in the ninhydrin reaction has been made the basis of quantitative procedures (Van Slyke, MacFadyen & Hamilton, 1941; Van Slyke, Dillon, MacFadyen & Hamilton, 1941); these have the advantages that, since simple dipeptides evolve no carbon dioxide, amino acids are specifically determined. A disadvantage of these methods is the laborious and time-consuming technique required. Methods based on the formation of copper complexes (Spies & Chambers, 1951; Spies, 1952) are imprecise because, even under the most favourable conditions, dipeptides give 70–80% of the extinction produced by their constituent amino acids. The Van Slyke nitrous acid technique has many disadvantages, notably the time required per determination and the non-stoichiometric reaction.

In view of these difficulties it was considered desirable to explore the possibility of utilizing other reactions for analytical purposes.

Smith (1948) observed that, when glycylglycine was incubated for several hours at room temperature and pH 8 with Co^{2+} ions, a pink colour developed which was more intense than the colour produced by an equivalent quantity of glycine. The reaction is not restricted to glycylglycine and similar colour production from other dipeptides

has been observed. Gilbert, Otey & Price (1951) made the significant discovery that atmospheric oxygen is involved in the colour-forming reaction and that the coloured compound is an oxygen-containing cobaltous complex. Suitable conditions have been found for the utilization of this reaction as a quantitative analytical method by using hydrogen peroxide as oxygenating agent and a suspension of cobaltous phosphate in borate buffer as the source of cobaltous ions (Crook & Rabin, 1954). Techniques have been evolved for the determination of dipeptide hydrolysis at two levels: these are designated the 'macro' and 'micro' methods and the choice of either depends on the availability and cost of the substrates.

MATERIALS AND METHODS

Glycyl-DL-alanine, glycyl-L-tryptophan, DL-alanyl-DL-alanine, glycyl-DL-phenylalanine, glycyl-L-tyrosine and acetyl-glycine were obtained from L. Light and Co., Colnbrook, Bucks.

Glycylglycine, glycyl-DL-leucine, DL-alanylglycine, DL-leucylglycine and diglycylglycine were obtained from Roche Products Ltd., Welwyn Garden City, Herts.

Sarcosylglycine and *N*-ethylglycylglycine were prepared by the method of Levene, Simms & Pfaltz (1924) and reprecipitated twice by the addition of ethanol to an aqueous solution.

Glycyl-L-proline was prepared by the method of Bergmann, Zervas, Schleich & Leimert (1932). The amino acids used were commercial preparations available in this laboratory.

Absorption spectra were measured in a Unicam Model SP. 500 quartz spectrophotometer with cells of 1 cm. path length.

Colorimetric determinations were made in a Gallenkamp Direct-Reading colorimeter with cells of 1 cm. path length.

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