DISCUSSION

2,6-Dichlorobenzonitrile has been identified in extracts of field soil and cultures of *P. putrefaciens* to which α -amino-2,6-dichlorobenzaldoxime and other 2,6-dichlorobenzaldoximes had been added. It has been claimed that *trans*-2,6-dichlorobenzaldoxime inhibits germination of seeds more actively than does the *cis*-isomer (Belgian Patent, 1960). As inhibition of seed germination is one of the characteristic properties of 2,6-dichlorobenzonitrile (Barnsley & Rosher, 1961; Koopman & Daams, 1960) it was expected that 2,6-dichlorobenzonitrile would be formed at different rates from two isomers as Hantzsch & Werner (1890) found for the formation of benzonitrile from unsubstituted benzaldoxime. However, no difference in the rate of formation of 2,6-dichlorobenzonitrile was detected.

Indolylpyruvic acid oxime is active in the *Avena* straight-growth test (Dannenberg & Livermann, 1957), presumably by conversion via indolylacetonitrile into indolylacetic acid within the plant (Ahmad & Spenser, 1960), but neither *Avena sativa* nor any of the other seeds germinated in sterile culture converted 2,6-dichlorobenzaldoxime or α -amino-2,6-dichlorobenzaldoxime into 2,6-dichlorobenzonitrile. Breakdown of α -amino-2,6-dichlorobenzaldoxime and 2,6-dichlorobenzaldoxime was rapid in non-sterile Steinberg solution and slow in the partially sterile one. Growth of *Salvinia auriculata* was stopped immediately in the former but continued in the partially sterile solution for a few days. It is probable, therefore, that micro-organisms formed most, if not all, of the 2,6-dichlorobenzonitrile.

Rates of formation of 2,6-dichlorobenzonitrile/mg. of nitrogen in soil (12 μ g./mg. of nitrogen/hr.) were many times greater than the maximum values for breakdown in the cauliflower homogenate (less than 0.1 μ g./mg. of nitrogen/hr.) calculated from

the sensitivity of the detection apparatus. As only part of the soil nitrogen is present in living micro-organisms, it appears that the conversion of 2,6-dichlorobenzaldoximes is brought about by micro-organisms and not, or very slowly, by higher plants.

SUMMARY

1. Soil micro-organisms convert a range of 2,6-dichlorobenzaldoximes into 2,6-dichlorobenzonitrile.
2. Attempts to demonstrate conversion by plants and a free-living nematode failed.
3. *Pseudomonas putrefaciens* forms 2,6-dichlorobenzonitrile from α -amino-2,6-dichlorobenzaldoxime and 2,6-dichlorobenzaldoxime.
4. There is no significant difference between the rates of formation of 2,6-dichlorobenzonitrile from *cis*- and *trans*-2,6-dichlorobenzaldoxime.

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The Presence of Acetyl Groups in Histones

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The *N*-terminal amino acids of calf-thymus histones have been found to be chiefly proline and alanine (Phillips, 1957, 1958), and this has been confirmed by Luck, Rasmussen, Satake & Tsvetikov (1958) and by Bisert & Sautière (1958).

When the histones were fractionated with sodium chloride and ammonia, or by the method of Daly & Mirsky (1955), it was found that the proline end groups were associated with the slightly lysine-rich part and the alanine end groups with the arginine-

rich part (Phillips, 1958; Biserte & Sautière, 1958). However, when these proteins were further separated, fraction F2a, which forms the major part of the slightly lysine-rich histones (a group having a ratio of 0.9–4 moles of lysine to a mole of arginine), and fraction F1, the very lysine-rich group (lysine to arginine ratio 4–15:1), both gave small yields of *N*-terminal amino acids. These amounted to 1 mole in 40 000–200 000 g. of the protein (Johns & Butler, 1962; Phillips, 1962). The observation of a low yield was first made with samples of very lysine-rich histone received from Professor J. M. Luck, and was unexpected since it had been reported that valine was the chief *N*-terminal amino acid in this fraction and was present to the extent of 1 mole in 7600 g. of protein (Luck *et al.* 1958).

On the other hand, the arginine-rich group (F3) has given alanine *N*-terminal groups amounting to about 1 mole in 16 000–18 000 g. of histone (Johns, Phillips, Simson & Butler, 1960), and the fraction F2b of the slightly lysine-rich histones has given 0.8 mole of proline and 0.2 mole of other amino acids as *N*-terminal groups in 15 000 g. of the protein (Johns & Butler, 1962).

Physical measurements have shown that the calf-thymus histones have molecular weights in the range 8000–51 000 (Butler, Davison, James & Shooter, 1954; Luck *et al.* 1956; Ui, 1957; Trautman & Crampton, 1959). In particular, the very lysine-rich group has the lower values 8000–10 000. This group also has other properties generally associated with small proteins, such as the need for a high concentration of trichloroacetic acid for precipitation (Luck *et al.* 1958) and solubility in 0.75*N*-perchloric acid (Johns & Butler, 1962). Thus the *N*-terminal amino acids in the two fractions F1 and F2a appear to be masked in some way or consist of labile or unusual amino acids. Some of these possibilities have been examined, and it now seems likely that the terminal amino acids are acetylated in some or all of the components of the fractions F1 and F2a. Some of the components of the fractions F2b and F3 also contain acetyl groups.

A preliminary account of this work has been given (Phillips, 1961).

MATERIALS AND METHODS

The whole histones were prepared as described by Phillips & Johns (1959*a, b*). The very lysine-rich fraction was prepared in two ways: (a) by chromatography on carboxymethylcellulose of the 10% ethanol-HCl extract from calf thymus (Johns *et al.* 1960), but with a phosphate-citrate buffer, pH 4.4, used instead of the usual acetate buffer for the elution; (b) by the perchloric acid-extraction method of Johns & Butler (1962). Samples of the very lysine-rich histone (fraction I of Luck *et al.* 1958; Satake, Rasmussen & Luck, 1960) and of whole histone were also kindly given by Professor J. M. Luck.

The slightly lysine-rich fractions F2a and F2b were prepared as described by Johns & Butler (1962) and the arginine-rich group F3 by the ethanol-HCl method of Johns *et al.* (1960). It was reprecipitated before use.

Acetyl groups. The substituents were determined as *N*-2,4-dinitrophenyl acetylhydrazide by a method involving hydrazinolysis of the protein and subsequent coupling with fluorodinitrobenzene at pH 3. The values for acetyl and propionyl groups obtained were corrected by assuming the same overall recovery of 70% found for ovalbumin and some acyl amino acids (Phillips, 1963). Acetate was also detected after partial hydrolysis of the histones in 1*N*- or 6*N*-H₂SO₄ at 100° for 5 hr. Ether extracts of these hydrolysates were chromatographed under conditions similar to those described by Brown & Hall (1950) for detecting aliphatic acid anions, and also by the method of Satake & Seki (1950) after treatment with hydrazine at 100° for 16 hr.

Other analyses. Hexose was estimated by the anthrone method of Hewitt (1958) with a glucose standard. Glucosamine was determined by the method of Rondle & Morgan (1955), ribose by the method of Meijbaum (1939) and deoxyribose by the method of Burton (1956). The *N*-terminal amino acids were determined by the modification of Sanger's (1945) fluorodinitrobenzene method described by Phillips (1958) and Phillips & Johns (1959*a, b*). Total amino acids were measured by a modification of Levy's (1954) method described by Phillips & Johns (1959*a, b*).

RESULTS

New observations on the identity and yield of *N*-terminal amino acids in the whole histones and histone fractions prepared in various ways are given in Table 1. Fractions F2b and F3 each have virtually a single *N*-terminal amino acid. In the other fractions, F1 and F2a, there are several *N*-terminal amino acids, each in relatively small amount, and moreover the yield of all such groups is very low and varies somewhat with the mode of preparation.

Some experiments are described below that were done to establish the reason for these low yields. It was assumed that these proteins have much lower molecular weights, as suggested by the values from physical measurements.

More vigorous coupling with fluorodinitrobenzene. To exclude the possibility that the low yield of end amino acids is due to an abnormally low rate of reaction with fluorodinitrobenzene, whole histone was allowed to react for 17 hr. at 50° instead of at room temperature. This modification produced no significant difference in the *N*-terminal amino acids and no change was produced when the coupling at 20° was carried out in the presence of 75 mM-sodium ethylenediaminetetra-acetate, which would remove, for example, copper atoms chelated to the *N*-terminal amino groups of the proteins. Measurement of the uncoupled lysine in a sample of very lysine-rich histone after dinitrophenylation at 20° showed, as was found previously for whole histone

Table 1. *N*-Terminal amino acids of histones

CMC, Carboxymethylcellulose.

Histone	Wt. of histone containing 1 mole of all <i>N</i> -terminal amino acids (g.)	Chief <i>N</i> -terminal amino acids†
F1 (very lysine-rich), CMC-column chromatography	37 000–61 000	Proline, alanine, lysine
Amberlite IRC-50 column chromatography*	56 000–270 000	Alanine, lysine, serine
Perchloric acid method	49 000–68 000	Glycine, proline, serine
F2a (slightly lysine-rich), acid-ethanol method	63 000–149 000	Alanine, glycine, proline, lysine
F2b (slightly lysine-rich)	15 000–17 000	Proline, 77–82 %
F3 (arginine-rich), acid-ethanol method	16 000–19 000	Alanine, 95–99 %
Whole histone (preparations H52–H55)	27 000–30 000	Proline, 43 %, alanine, 37 %
(from Professor Luck)	25 000	Proline, 37 %, alanine, 41 %

* Samples from Professor J. M. Luck.

† Where proportions are given they are molar percentages of all the *N*-terminal amino acids found.

(Phillips, 1958), that the ϵ -amino groups at least were completely coupled.

Unusual amino acids as N-terminal groups.

(a) Sarcosine. 2,4-Dinitrophenylsarcosine (DNP-sarcosine) travels at the same position as DNP-proline in the chromatographic system used, and it too has an optical spectrum with a maximum near 375 m μ , compared with the maximum at 360 m μ of dinitrophenylamino acids such as DNP-alanine. Moreover, DNP-sarcosine is readily broken down by hot acid and in 2.5 hr. at 100° in 11*N*-hydrochloric acid is all degraded to DNP-glycine, dinitrophenol and free sarcosine. However, terminal DNP-glycine is absent from the very lysine-rich histones when prepared by chromatography on Amberlite IRC-50 or on carboxymethylcellulose, and with histone F2a glycine amounts at the most to 25 % of the already small content of *N*-terminal amino acids.

(b) Pyrrolidonecarboxylic acid. This acid has been sought by partial hydrolysis of very lysine-rich histone in 0.3*N*-hydrochloric acid at 100° for 1 hr. It was shown by Bethke & Steenbock (1923–24) that 50 % of pyrrolidonecarboxylic acid is hydrolysed to glutamic acid under these conditions. However, with the histone, glutamic acid accounted for about 2 % of the *N*-terminal groups after this treatment and only 4 % appeared after hydrolysis for 2.5 hr.

Other labile N-terminal groups. Samples of very lysine-rich histone, which gave yields of about 1 mole of *N*-terminal amino acids in 240 000 g. of protein, gave 1 mole of 2,4-dinitroaniline in 42 000 g. of the histone. However, at least half of this dinitroaniline was derived from the ϵ -DNP-lysine, which is degraded to the extent of about 0.5 % under these conditions.

Nucleotide end group. The optical spectra of the histones (Davison, James, Shooter & Butler, 1954; Cruft, Mauritzen & Stedman, 1957; Ui, 1957) make it very unlikely that there is a nucleotide or similar

compound as an integral part of the calf-thymus histones, and this is supported by results with some fractions examined here. A sample of fraction I (very lysine-rich) from Professor J. M. Luck had a maximum extinction in acid at 274 m μ and in alkali at 286 m μ . The value in acid corresponds to 1 mole of tyrosine in 21 000 g. of protein, which is very close to the analytical value for tyrosine given by Luck *et al.* (1958). Samples of the slightly lysine-rich fraction F2a had maxima in acid at 275–276 m μ and extinctions corresponding to 3.1–3.5 moles of tyrosine/100 moles, whereas the analytical values were 2.1 and 2.9 moles of tyrosine/100 moles as determined by the dinitrophenyl-derivative method. This fraction also contains only a trace of phosphorus, amounting to 1 g. atom in 170 000 g. of protein.

Carbohydrate content. A carbohydrate moiety could mask a *N*-terminal amino acid and moreover any acyl groups found might be attached to the carbohydrate rather than to α -amino groups. Various methods for measuring carbohydrates have shown that whole histone gave negligible reactions for deoxy-sugar, pentose and glucosamine. The hexose content by the anthrone method was equivalent to 1 mole of glucose in 100 000–160 000 g. of histone. With the histone fraction F1, prepared by column chromatography, and F2a and F3 prepared by the acid-ethanol-extraction method, values as low as 1 mole of glucose (equivalent) in 160 000–215 000 g. of protein have been obtained. On the other hand, the very lysine-rich histone prepared by perchloric acid-extraction method appears to contain extraneous carbohydrate since it has given the equivalent of 1 mole of glucose in 12 000 g. The tint of colour developed with all the histones in this method is the same as that given by glucose and unlike that given by mannose or ovalbumin.

Acyl groups in histones. Acetate was detected in the sulphuric acid hydrolysates of the very lysine-rich and slightly lysine-rich histones by

Table 2. *Acetyl groups and total N-terminal groups in histones*

Histone	Wt. of histone containing 1 mole of acetyl groups (g.)	Wt. of histone containing 1 mole of N-terminal groups (amino acid + acetyl) (g.)*	Proportions of all N-terminal groups as % of total*	
			Acetyl	Amino acid
Whole histone	19 000–25 000	11 000–13 500	54–58	42–46
Whole histone (dinitrophenylated)†	20 000	11 000	53	47
Whole histone (hydrazinolysed for 16 hr. at room temp.)	350 000	—	—	—
Very lysine-rich group F1 (perchloric acid preparation)	15 000–18 000	12 000–14 000	73–80	20–27
Slightly lysine-rich group:				
F2a	10 000–14 000	9 000–12 500	85–92	8–15
F2a (tryptic digest), soluble peptides	9 000	—	—	—
F2a (tryptic digest), insoluble precipitate	50 000	—	—	—
F2b	32 000–38 000	10 000–11 000	29–32	68–71
Arginine-rich group: F3	21 000–28 000	10 000	36–48	52–64

* Assuming that the acetyl groups found here are N-terminal groups (see text).
† Corrected for the content of dinitrophenyl groups.

Fig. 1. Formation of acethydrazide (●) and propionic hydrazide (○) during the hydrazinolysis of whole calf-thymus histone at 100°. Each reading is derived from 2.5 mg. of histone and the hydrazides were measured as 2,4-dinitrophenyl derivatives at 420 mμ in 5.5 ml. final volume.

ovalbumin (Narita, 1961) and in cytochrome *c* (Titani, Yaoi, Narita & Okunuki, 1962) was achieved by the prior separation of an acyl peptide from the proteins after digestion with enzymes. This hydrazinolytic method could not be applied to an intact protein, since the very large amounts of amino acid hydrazides formed interfere with the detection of the acyl hydrazide. Whole histone and the fractions from it were therefore examined by another method developed with ovalbumin (Phillips, 1963) and the results are given in Table 2 and Fig. 1.

As with ovalbumin, the formation of acethydrazide rises to a maximum in 15–20 hr. of hydrazinolysis at 100° and thereafter declines slowly. The formation of propionic hydrazide (measured as *N*-2,4-dinitrophenylpropionic hydrazide) is also shown in Fig. 1.

Hydrazinolysis of calf-thymus histone at room temperature yielded only a trace of acethydrazide (1 mole in 250 000 g. of protein in 2 days) and no propionic hydrazide was formed.

Table 2 shows also the weights of histone or histone fraction containing 1 mole of all *N*-terminal groups (*N*-terminal amino acids + acetyl groups), assuming that the acetyl groups are in fact *N*-terminal. The proportion of terminal groups of each type in each fraction is also expressed as a percentage of the combined terminal groups.

A preparation of acylase (kindly given by Dr R. Wade), which readily hydrolysed acetyl-L-methionine, was apparently without effect on the slightly lysine-rich histone fraction F2a at pH 7. This was demonstrated by the absence of any definite change in the *N*-terminal amino acid analysis of the sample after incubation for 24 hr. with the enzyme at 38°.

using a modification of the method of Brown & Hall (1950) for the paper chromatography, but neither this method nor the conversion of the extract into hydrazides and chromatography under the conditions of Satake & Seki (1950), could be made quantitative. The demonstration by a hydrazinolytic method of *N*-terminal acetyl groups in tobacco-mosaic-virus protein (Narita, 1958*a, b*), α -melanocyte-stimulating hormone (Harris, 1959),

DISCUSSION

The very lysine-rich group of histones, F1, amounts to about 20% of the whole histone of calf thymus and the slightly lysine-rich fraction, F2a, amounts to about 35%. The experiments described above made it seem unlikely that a large proportion of the *N*-terminal amino acids in these major histone fractions is masked by a nucleotide or similar compound, or by carbohydrate, or that they fail to react with fluorodinitrobenzene. Previous work had shown that more vigorous hydrolysis failed to increase the *N*-terminal amino acids appreciably (Phillips, 1958). Moreover, the presence of terminal sarcosyl and pyrrolidone-carboxylic acid residues can be ruled out. Furthermore, there was no marked change in the *N*-terminal groups when the reaction with fluorodinitrobenzene was carried out in 6*M*-guanidium chloride (Phillips, 1958; Phillips & Johns, 1959*a, b*). On the other hand, all the histone fractions have yielded acetyl groups (Table 2), the fractions F1 and F2a giving more and the fractions F2b and F3 giving less than that obtained from whole histone. It is suggested that these groups are *N*-terminal substituents in some of the fractions of calf-thymus histones.

Propionic hydrazide is apparently formed from the degradation of cysteine and cystine by hydrazine (Phillips, 1963), and hydrogen sulphide can be detected in the acidified hydrazinolysates of the histone fractions. The cystine content of the histones studied here is unknown, though it is probably very low (see Crampton, Moore & Stein, 1955; Crampton, Stein & Moore, 1957), and in all the fractions the yield of propionic hydrazide is less than the amount of acetylhydrazide formed. With other proteins, the yield of propionic hydrazide after 17 hr. of hydrazinolysis at 100° was variable. With ovalbumin, it was equivalent to about 20% of the cysteine + cystine content of the protein, and with chymotrypsin the value was equivalent to only 10%. Thus it has not yet been established whether there are propionyl groups in the intact histones or not.

Starch-gel electrophoresis of these histones (Johns, Phillips, Simson & Butler, 1961; Johns & Butler, 1962) has shown that only fraction F2a gives a single band on the gel, whereas F2b gives two bands, F1 gives three bands and the arginine-rich group F3 gives two major and two minor bands. An appreciable acetyl-group content in the arginine-rich fraction and in the slightly lysine-rich fraction F2b was unexpected, since these fractions had already given a yield of *N*-terminal alanine and proline respectively of 1 mole in 15000–18000 g. However, the complexity shown by the gel electrophoresis could well account for the presence of

both amino acid and acetyl *N*-terminal groups. If the acetyl groups are indeed *N*-terminal then in the calf-thymus histones there are at least four proteins bearing them.

Acetyl *N*-terminal groups have already been reported in tobacco-mosaic-virus protein (Narita, 1958*a, b*), α -melanocyte-stimulating hormone (Harris, 1959), ovalbumin (Narita, 1961; Marshall & Neuberger, 1961) and cytochrome *c* (Titani *et al.* 1962). In ovalbumin, which has four acetyl groups per molecule, it is probable that three are present as *N*-acetylglucosamine residues (Johansen, Marshall & Neuberger, 1960), but the virtual absence of hexosamine from the histones shows that the acetyl groups in this case cannot be present in this form. The stability of the bonding of acetyl groups in the histones towards hydrazine at room temperature implies that they are present as *N*-acetyl rather than as *O*-acetyl structures.

The final confirmation of the position of these groups in the histones must await the isolation of acetylpeptides. Preliminary studies with the histone fraction F2a show that, on digestion with trypsin, the acetyl groups remain in the small soluble peptides and not in the precipitate that forms during the digestion. The failure of an acylase preparation to alter the *N*-terminal amino acid content of the slightly lysine-rich fraction F2a may only reflect the inability of this enzyme to act in the absence of a nearby free α -carboxyl group on its substrate.

With acetyl groups as well as amino acids as *N*-terminal groups, the average molecular weights of the histones per mole of terminal groups in all the fractions lie in the range 9000–14000 (Table 2). This is near to the most recent values suggested by physical measurements (Trautman & Crampton, 1959).

SUMMARY

1. The very lysine-rich (F1) and part (F2a) of the slightly lysine-rich histones of calf thymus give a very small yield of *N*-terminal amino acids by the fluorodinitrobenzene method.
2. This is apparently not due to masking of the *N*-terminal group by nucleotide or carbohydrate, nor to aggregation or incomplete reaction, and *N*-terminal sarcosyl and pyrrolidonecarboxylic acid residues are not present.
3. Acetyl groups have been found in these fractions amounting to about 1 residue in 15000–18000 and 10000–14000 g. of protein in F1 and F2a respectively.
4. The other slightly lysine-rich histones (F2b) and the arginine-rich group (F3) also contain some acetyl groups, amounting to 1 residue in 32000–38000 and 26000–28000 g. of protein respectively.
5. The acetyl groups are probably *N*-acetyl, and

it is suggested that they are the *N*-terminal substituents of at least four proteins in calf-thymus histones, namely fraction F2a and part of the groups F1, F2b and F3.

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all-*cis*-Docosa-4,7,10,13,16,19-hexaenoic Acid in Ox Retina

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The polyenoic acid present in ox retina reported by Hörhammer, Wagner & Wolff (1960) was shown by Bartley, van Heyningen, Notten & Renshaw (1962) to be a C₂₂ acid, probably with six double bonds/molecule. This finding has been confirmed and the positions of the double bonds have been determined by degradation.

MATERIALS, METHODS AND RESULTS

Isolation of methyl docosaheptaenoate. Eyes from cattle of various ages and breeds and both sexes were dissected as soon as possible after removal and the retinae were stored at -15°. Retinae (18 g.), 20% (w/v) potassium hydroxide solution (45 ml.) and methanol (45 ml.) were warmed at 60° for 3 hr. under nitrogen with occasional shaking. The cooled