of that obtained with an equal weight of *N*-acetylglucosamine.

4. The *N*-acetylhexosamines after treatment with alkali show a single maximum absorption at 230 mμ. and after the addition of the *p*-dimethylaminobenzaldehyde reagent the purple colour which develops shows two absorption maxima, at 550 and at 590 mμ.

5. A mixture of glucose and lysine heated with sodium carbonate shows no characteristic absorption maximum at 230 mμ. The purple colour obtained on the subsequent addition of *p*-dimethylaminobenzaldehyde reagent shows a single absorption maximum at about 560 mμ.

6. The human blood-group mucoids yield between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine, and there is no enhanced colour production when a borate buffer is employed. The absorption characteristics of the chromogenic substances which arise from the group substance after heating with alkali, and of the coloured product obtained on the addition of the *p*-dimethylaminobenzaldehyde reagent support the belief that the colour given by the group substances arises from reducing *N*-acetylchondrosamine end groups and closely linked *N*-acetylglucosamine residues, which are readily liberated by the action of alkali.

7. The A substance, after oxidation with hypoiodite, gives rise to a coloured complex on treatment with alkali and *p*-dimethylaminobenzaldehyde which shows a reduced absorption intensity compared with that given by the unoxidized material. A substance oxidized with periodate and treated under similar conditions shows no decrease in absorption. The significance of these findings is discussed.

The investigation was supported by personal grants made to two of the authors (W.M.W. and D.A.) by the Medical Research Council and the Governing Body of the Lister Institute respectively. The authors are indebted to the University of London Research Fund for a grant to purchase the Uvispek spectrophotometer used. We wish to acknowledge our indebtedness to Miss V. Hayes and Miss J. Pugh for valuable technical assistance.

REFERENCES

Adams, R. & Coleman, G. H. (1944). *Organic Syntheses*, p. 214. New York: Wiley.
Aminoff, D. & Morgan, W. T. J. (1949). *Biochem. J.* **44**, xxi.
Aminoff, D. & Morgan, W. T. J. (1951). *Biochem. J.* **48**, 74.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
Annison, E. F. & Morgan, W. T. J. (1951). *Biochem. J.* **49**, xxiv.
Blumlein, O. F. (1884). *Ber. dtsch. chem. Ges.* **17**, 2578.
Cornforth, J. W. & Cornforth, R. H. (1947). *J. chem. Soc.* p. 96.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Ehrlich, P. (1901). *Dtsch. med. Wschr.* **15**, 434, 498.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Gottschalk, A. & Lind, P. E. (1949). *Nature, Lond.*, **164**, 232.
Gottschalk, A. & Partridge, S. M. (1950). *Biochem. J.* **46**, vi.
Lewy, M. (1887). *Ber. dtsch. chem. Ges.* **20**, 2576.
MacLeod, M. & Robison, R. (1929). *Biochem. J.* **23**, 517.
Morgan, W. T. J. (1938). *Chem. & Industr.* **57**, 1191.
Morgan, W. T. J. (1946). *Biochem. J.* **40**, xv.
Morgan, W. T. J. (1947). *Experientia, Basel*, **3**, 257.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
Morgan, W. T. J. & Waddell, M. B. R. (1945). *Brit. J. exp. Path.* **26**, 387.
Müller, F. (1901). *Z. Biol.* **42**, 468.
Osaki, S. & Turumi, K. (1947). *Tohoku J. exp. Med.* **49**, 11.
Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.
Stolte, K. (1908). *Beitr. chem. Physiol. Path.* **11**, 19.
Vasseur, E. & Immers, J. (1949). *Ark. Kemi*, **1**, 253.
Vogel, A. I. (1939). *Quantitative Inorganic Analysis*. London: Longman, Green and Co.
White, T. (1940). *J. chem. Soc.* p. 428.
Zuckerkandl, F. & Messiner-Klebermass, L. (1931). *Biochem. Z.* **263**, 19.

A Study of the Breakdown of Ribonucleic Acid in Tobacco-leaf Extracts

By G. PARKER*
Rothamsted Experimental Station, Harpenden, Herts

(*Received* 18 *October* 1951)

There is an extensive literature on the preparation of ribonucleic acids and their degradation products from animal tissues and micro-organisms. By contrast, apart from the isolation of certain plant virus nucleoproteins, little work has been undertaken until recently, on the preparation of ribonucleic acids from plant material.

* Present address: The Distillers Co. Ltd. Speke, Liverpool 19.

Foreman (1938) suggested the presence of nucleoprotein in leaves of perennial rye grass, to explain the fact that cytolysed leaves yielded only 30 % of their phosphorus on extraction with boiling water, whereas leaves previously dried at 85° yielded all their phosphorus by cold water extraction. The occurrence in, and isolation of, ribonucleic acid from leaves of barley, rye and spinach has been reported by von Euler & Hahn (1947, 1948). These

workers used an alkaline extraction method similar to that employed by Schmidt & Thannhauser (1945) for animal tissues. Takasugi (1943, 1944) used 10% sodium chloride solution to extract the nucleic acids from barley roots. Ogur & Rosen (1950) used perchloric acid for the separate extraction of ribo- and deoxyribo-nucleic acids from corn root tips.

Pirie (1950) has described the isolation of a ribonucleoprotein from the sap of the leaves of healthy tobacco plants. As prepared, this material, whose concentration in sap varies with the age of the leaf, is associated with enzymes (ribonuclease and phosphatase) capable of bringing about its degradation. Pirie was able to obtain the nucleic acid from fresh preparations of this nucleoprotein by denaturation of the protein with trichloroacetic acid (TCA), but he could only isolate degradation products of the acid after such precipitation from sap which had not been ultracentrifuged. It appeared of interest to examine these degradation products more closely, and the work reported in the present paper deals with the preparations of various phosphorus-containing fractions obtained from sap by chemical and enzymic degradation of the nucleic acid moiety of the trichloroacetic acid sap precipitate which contains this nucleoprotein.

MATERIALS AND GENERAL PROCEDURES

Preparation of sap-trichloroacetic acid precipitate and of chloroplast-trichloroacetic acid precipitate

Leaves, between 10 and 20 cm. long, of tobacco (*Nicotiana tabacum* var. White Burley) from plants grown in a heated glasshouse were used for most of the work. Single experiments confirming the results obtained with tobacco have also been carried out with the leaves of turnip, spinach, French bean, comfrey, snowberry and grass, and oat and barley seedlings. Unless stated otherwise, results quoted in the text refer to tobacco. The leaves were taken at all periods of the year; different batches consequently probably differed in age, but no attempt has been made to analyse the results for possible seasonal variations in composition.

The midribs were removed from the leaves and the laminae passed through a domestic mincer and squeezed on madapollam into a receiver cooled in ice. The residue was re-minced and squeezed again. Cell debris, chloroplasts and starch granules were removed by centrifuging for 15 min. in a chilled angle centrifuge at 8000 rev./min. (6400*g*). The supernatant fluid, which is subsequently referred to as sap, was stored at 4° and used within 30 min. of preparation. Unless otherwise stated, it was treated with 50% (w/v) trichloroacetic acid (TCA) to a final concentration of 5%. The precipitate was centrifuged down at once and washed several times, first with 2% (w/v) TCA and then with water, the volume of wash liquor in each case being about half that of the sap, until the washings contained only 1–2 μg. P/ml. The solid residue is subsequently termed sap-TCA precipitate.

The chloroplasts, separating from crude sap as a sticky layer overlying the starch, were roughly separated from the latter by scraping with a spatula. They were washed twice with distilled water and were then dispersed to give a suspension which was used immediately after preparation. It was precipitated with TCA and washed in the same way as the sap, and is referred to subsequently as chloroplast-TCA precipitate.

Analytical methods

Phosphorus. This was determined by a modification of the method of Kuttner & Lichtenstein (1932). Inorganic phosphate values were obtained on samples which had not been incinerated. P retained by samples containing Ba was estimated by incineration with H_2SO_4 until the sample dissolved completely: after dilution, the precipitate was centrifuged down, and the inorganic phosphate content of the supernatant liquid measured.

Ribose. Total ribose estimations were made by the orcinol method of Pirie (1936) using ribose standards. The colour developed was measured in a photoelectric absorptiometer (the Evans Electroselenium Portable model was used) using Ilford filter 622.

Values for purine-bound ribose were obtained by the Markham & Smith (1949) modification of Militzer's (1946) orcinol method. The colour developed was read in the absorptiometer against that due to ribose standards, using Ilford filter 205.

Purines. These were determined in N-H_2SO_4 hydrolysates by the following methods.

Adenine. This was estimated colorimetrically by Woodhouse's (1950) method, modified by the use of twice the concentration of ammonium sulphamate recorded by Woodhouse. This avoided difficulties, otherwise encountered, through incomplete decomposition of excess nitrite, which led to production of cloudy solutions.

Guanine. The method of Williams (1950) was employed. Standard guanine solutions were subjected to precipitation along with the samples, and the colour developed with the phenol reagent was read, using Ilford filter 608.

Total pyrimidines. These were obtained in the free form by the method of Daly, Allfrey & Mirsky (1950), and were then determined by the colorimetric procedure of Soodak, Pircio & Cerecedo (1949) using standard uracil solutions and Ilford filter 608.

Phosphatase activity. This was determined using, as substrate, solutions of sodium β-glycerophosphate and metaphosphate, and yeast adenylic acid. The reaction mixture comprised substrate solution at pH 6, 0·02 M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total volume of 5 ml., and contained 100 μg. P/ml. It was incubated at 37°. Samples were withdrawn at intervals and pipetted into an equal volume of 10% (w/v) TCA: after centrifuging at 1500 rev./min. for 10 min., the supernatant fluid was poured off and analysed for inorganic phosphate. Control reaction mixtures, from which the enzyme preparations and the substrate were individually omitted, were set up initially. They were found to give such low blank values as to be unnecessary, and were omitted in the later experiments.

Ribonuclease activity. This was measured by determining the amount of P becoming soluble in the uranyl nitrate and TCA reagent of McFadyen (1934) after action of the enzyme preparation on yeast ribonucleic acid. The reaction mixture comprised a solution of the acid brought to pH 6, purified by

the method of Vischer & Chargaff (1948*b*), 0·02M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total vol. of 5 ml., and contained 100 μg. P/ml. It was incubated at 37°. Samples were removed at intervals into 1 ml. of the uranyl reagent, chilled at 0° for 15 min. and then centrifuged at 1500 rev./min. for 10 min.; the supernatant fluid was poured off and an appropriate volume taken for total P analysis. As in the case of the phosphatase determinations, control reaction mixtures omitting enzyme or substrate were found to be unnecessary, and were only set up in a few preliminary experiments.

The optimum pH for nuclease activity was obtained in a similar reaction mixture, using the Michaelis (1931) veronal-acetate buffer (1·0 ml. of the diluted stock solution in 5 ml. total reaction mixture) of appropriate pH. The pH of the reaction mixture was determined at the beginning and the end of the experiment, but showed no signs of drift during the reaction at any pH employed.

Paper chromatography. Purines and pyrimidine nucleotides were separated on Whatman no. 1 or no. 11 paper using the *tert.*-butanol-HCl solvent system of Smith & Markham (1950). They were located on the paper by the mercury reagents of Vischer & Chargaff (1948*a*) and by viewing in suitably filtered ultraviolet light (Holiday & Johnson, 1949).

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph.

pH *measurements.* These were made with a glass electrode.

RESULTS

Fractionation of sap-TCA precipitate: preparation and properties of mononucleotide solutions

Sap-TCA precipitate, prepared as described, was mixed with water to a thin paste, cooled to 0° and brought to pH 9 with 0·5N-NaOH. Partial solution occurred. After standing overnight at 4°, the mixture was centrifuged at 8000 rev./min. (6400*g*) and the supernatant decanted. The residue was re-extracted with NaOH, pH 9, at 4° for several hours, again centrifuged down and then washed with a little water. The combined dark brown extracts, the total volume of which was approximately one-third that of the original sap, contained much of the protein originally present in the sap-TCA precipitate. This was thrown out by progressive acidification to a pH of approximately 6, 5 and 4. At each pH the precipitate was centrifuged down and washed with a little water, the washings being added to the main supernatant before the pH was readjusted. The almost colourless pH 4 supernatant contained no nucleic acid as it gave no precipitate on acidifying to pH 1 or less. It was treated with saturated $Ba(OH)_2$ solution to pH 9 and stood overnight at 4°. Any precipitate separating was centrifuged out, and the nucleotides were precipitated from the supernatant with 3 vol. of 95% ethanol at 4°. The barium salts were dissolved in the minimum volume of water and decomposed with 0·5N-H_2SO_4 to pH 3. Precipitated $BaSO_4$ was centrifuged down and washed with a little water, and the combined supernatants were treated with a little $Ba(OH)_2$ solution and quantitatively freed from Ba^{++} ions with 5% (w/v) Na_2SO_4 before finally centrifuging clear. The aqueous ethanolic supernatant remaining after separation of the barium nucleotides usually yielded a second crop of barium salts when treated with more $Ba(OH)_2$ solution and stored at 4° for 24 hr.: these were decomposed with H_2SO_4 in the same way.

In Table 1 is given the distribution of P between the residue insoluble at pH 9, the nucleotide preparations and the protein precipitates in the above procedure. These three fractions comprise the bulk of the P originally present in the sap-TCA precipitate. The remaining 10% was mostly found

Table 1. *Distribution of phosphorus in some fractions of sap*

	P, as % of total of		
Fraction	Sap	Sap-TCA precipitate	pH 9 extracts
pH 9-insoluble residues	1–3	5–15	—
Mononucleotide preparations	7–10	40–55	50–65
Protein precipitates	3–8	20–35	20–40

associated with the $BaSO_4$ precipitates obtained by decomposition of the barium nucleotides. Small amounts were also found in the precipitates sometimes obtained with Ba^{++} at pH 9 before addition of ethanol, and in the aqueous ethanolic supernatants remaining after precipitation of the 'second crop' nucleotides. In this way it is possible to account for a total of over 95% of the P of the sap-TCA precipitate.

In a single experiment, several of the aqueous ethanolic solutions remaining after precipitation of two crops of nucleotides were pooled and evaporated to one-tenth volume in vacuum, but attempted precipitation of further nucleotides by $Ba(OH)_2$ and ethanol yielded no barya-soluble, ethanol-insoluble material.

The figures in Table 1 vary somewhat with each batch of sap. This is not surprising. Variations are to be expected from causes such as differences in age of leaves taken at various times of the year, the use of different samples of soil in growing the plants, and unavoidable variations in the experimental technique with different lots of leaves. The values given are the extreme ranges obtained with several different batches of sap.

No precipitate was produced in the preparations by solutions of uranyl nitrate in TCA at a pH of less than 1, but addition of alkali caused mononucleotides to be thrown down, maximum visible precipitation occurring in the range pH 1·5–2 (cf. Zittle, 1946). Treatment with picric acid had no effect, but Buell's (1943) aluminium picrate reagent gave immediate yellow precipitates containing the adenylic acid and most of the guanylic acid of the preparations. Pyrimidine nucleotides are not precipitated by this reagent but their occurrence was indicated by the presence of a substantial proportion (50–60%) of the initial P in the filtrates. Confirmation was obtained in later experiments, when paper chromatography of preparations after 60 min. hydrolysis in N-HCl showed the presence of the four components, adenine, guanine, cytidylic and uridylic acids, typical of ribonucleic acids.

Other experiments of the type described, all carried out on 'first crop' nucleotide preparations, indicated that these contained more pyrimidine than purine bases. This was supported by analysis of the preparations for purine-bound ribose, which indicated that this accounted for some 40% of the total carbohydrate. Confirmation was obtained by analyses of the preparations for purine and pyrimidine content. Typical results are shown in Table 2, which also

Table 2. *Purine and pyrimidine composition of two nucleotide preparations*

(In exp. 1, 1 l. sap contained 191 mg. P. It gave a sap-TCA precipitate containing 27·3 mg. P, and yielded 15·3 mg. P in nucleotide preparations. In exp. 2, the sap had 179 mg. P/l. and gave a sap-TCA precipitate containing 45·3 mg. P and yielded 17·7 mg. P in nucleotide preparations. 'First crop' indicates nucleotides obtained from the initial barytasoluble, ethanol-insoluble precipitates; 'second crop' preparations resulted from addition of more $Ba(OH)_2$ to the supernatants from the 'first crop' precipitates. The composition of the nucleotide preparations is given as molecules base/100 atoms P.)

Exp.	Fraction	Adenine	Guanine	Total pyrimidines	P
		(molecules/100 atoms total P)			(atoms)
1	First crop	2·2	32·8	58·9	93·7
	Second crop	0·6	4·6	1·2	6·3
	Total	2·8	37·4	60·1	100
2	First crop	1·5	26·7	65·0	90·4
	Second crop	3·1	5·1	1·6	9·6
	Total	4·6	31·8	66·6	100

Table 3. *Effect of removal of lipids upon yields and composition of nucleotide preparations*

(Two equal portions of sap, from the same batch of leaves, were precipitated with TCA. The sap-TCA precipitates were fractionated for nucleotides as described in the text, one being treated with lipid solvents before fractionation. 'First' and 'second' crops have the meanings designated in Table 2. The composition of the preparations is given as molecules base/100 atoms P. Results of experiments on two separate lots of leaves are presented: (1) the sap (1 l., 231 mg. P) gave 45·5 mg. P in the sap-TCA precipitate. If lipids were not removed 21·2 mg. P were recovered in nucleotide preparations: if lipids were removed before fractionation, only 14·7 mg. P were so recovered; (2) the sap (1 l., 183 mg. P) gave 37·8 mg. P in the sap-TCA precipitate. This yielded 16·5 mg. P and 13·6 mg. P respectively as nucleotides following fractionation in presence and in absence of lipids.)

		Adenine		Guanine		Total pyrimidines		Phosphorus	
	Fraction	Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2
		(molecules/100 atoms total P)						(atoms)	
Lipids present	First crop	2·2	2·3	29·9	19·4	57·1	63·8	90·1	85·2
	Second crop	0·6	0·6	4·0	6·7	3·5	5·8	9·9	14·8
	Total	2·8	2·9	33·9	26·1	60·6	69·6	100	100
Lipids absent	First crop	0·4	0·2	32·5	30·4	42·7	52·8	78·2	86·9
	Second crop	3·9	1·7	10·5	6·4	7·0	4·9	21·8	13·1
	Total	4·3	1·9	43·0	36·8	49·7	57·7	100	100

shows that in the 'second crop' preparations this order is reversed and purines predominate. However, the total amount of pyrimidines in the 'first' and 'second' crops from any one batch of sap is always considerably greater than the total amount of purines.

Effect of ageing sap-TCA precipitate on phosphorus distribution

A small number of experiments was carried out on sap-TCA precipitates which had been left in contact with their TCA supernatants at room temperature for some hours before collection. The results were rather variable. However, the sap-TCA precipitate collected immediately after preparation usually contained somewhat more P than did the corresponding precipitate, from an equal volume of sap, allowed to age for some hours before it was collected. The difference increased the longer the precipitate was left to age. Aged precipitates yielded less P in the pH 9 extracts than did the fresh precipitates, but these extracts yielded protein precipitates which retained more P than did fresh sap-TCA precipitate extracts. Invariably, ageing of a sap-TCA precipitate led to reduction in the amount of P that was isolated in the nucleotide preparations.

Effect of removal of lipids from the sap-TCA precipitate upon yield and composition of the nucleotide preparations

Two equal volumes of a sap were precipitated with TCA and the precipitates washed in the usual manner. One precipitate was subjected to alkaline fractionation immediately. The other was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless: the residue was washed with water and then fractionated for nucleotides. Results of typical experiments are given in Table 3. This shows that removal of lipids from the sap-TCA precipitate lessened the amount of P which could be isolated as nucleotides. This was reflected in the total amount of pyrimidines present in the nucleotide preparations. The purine contents were affected to a very much smaller extent and the change may not be significant as it is difficult to ensure absolutely identical conditions of fractionation for each half of the batch of sap. The amount of P separating with the protein precipitates during fractionation was correspondingly greater in these cases than in the parallel experiments on precipitates from which the lipids were not removed. Removal of lipids from the sap-TCA

precipitate had no effect upon the amount of P which could be subsequently removed from the material insoluble at pH 9. This is described more fully in a subsequent section.

Further fractionation of pooled pH 9-*insoluble residues and protein precipitates*

In a few preliminary experiments, the residue remaining after two extractions of the sap-TCA precipitate was pooled with the protein precipitates and refractionated after a further TCA precipitation. Table 4 shows that some 70% of the P remained in the TCA supernatant. Most of the P precipitated was either not extracted at pH 9 or precipitated along with the proteins again, only 5–15% (i.e. 2–6% of the initial total) being finally obtained in nucleotide preparations.

Table 4. *Phosphorus distribution during fractionation of pooled* pH 9-*insoluble residues and protein precipitates*

(The figures refer to three separate fractionations of pooled material)

Percentage total P in:	
5% TCA supernatant	70·2, 71·4, 63·0
Nucleotide preparations	4·5, 1·9, 6·1
Percentage P of TCA precipitate in:	
pH 9-insoluble residues	38·6, 29·4, 51·6
Nucleotide preparations	14·9, 6·4, 11·5
Protein precipitates	25·2, 47·3, 22·7

Phosphorus content of pH 9-*insoluble residues*

The residue from sap-TCA precipitate after two extractions at pH 9 usually consisted of a gel which did not sediment cleanly at 8000 rev./min., and still contained 5–15% of the P of the sap-TCA precipitate. The material was difficult to mix effectively with extracting liquid, but 50–60% of its P could be removed by repeatedly washing with water (Table 5). If the material were given a preliminary treatment with 5% (w/v) TCA and then washed several times with water, a larger proportion (70–80%) was extracted. This is probably due to the change in texture of the material, on treatment with the TCA, to a hard, granular form, readily sedimenting at 1500 rev./min., for, in samples that had been washed several times with water until no more P was extracted, treatment of the residues with 5% (w/v) TCA liberated more P and in this way 70–80% of the original amount could again eventually be extracted.

Effect of incubation on the pH 9-*insoluble residues*

Increases in the amount of P that could be extracted by water from the pH 9-insoluble preparations, were observed on raising the temperature and extending the time of contact between the solvent and the sample. Lipid-free material incubated aseptically with water at 37° for 2–3 days yielded 80–90% of its P in water extracts. Samples thus treated did not liberate further P by subsequent treatment with TCA. The increased liberation is probably due to enzyme activity, as small amounts of inorganic phosphate were present in the extracts. Furthermore, when these extracts were treated with Buell's (1943) reagent the amount of P present in the precipitates increased with time to a maximum value after 2 days' incubation.

Dialysis was investigated as a means of separating nucleotides and nucleosides from undegraded ribonucleic acid and polynucleotide material following incubation of the pH 9-insoluble samples. Portions were suspended in water, adjusted to pH 7 and incubated at 37° for 24–36 hr.,

Table 5. *Liberation of phosphorus from* pH 9-*insoluble residues by further solvent action*

(Sap (1 l., 273 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (41 mg. P) was split into two equal portions, one of which was extracted with lipid solvents, before extraction at pH 9. The residues insoluble at pH 9 contained 2·5 mg. and 2·36 mg. P respectively. They were suspended in water and divided into two equal portions, and washed with water alone or with 5% (w/v) TCA followed by water. The amount of P extracted by these various treatments is recorded in the table as the % of the initial total of the precipitates. In the case of the lipid-free samples, these percentages have been calculated to include the phospholipid P removed from the sap-TCA precipitate before alkaline extraction.)

Previous treatment of sap-TCA precipitate	None	Extracted with lipid solvents
Washed with water alone		
Extract:		
Lipid solvent	—	5·7
1st water	49·2	44·2
2nd water	7·2	11·2
3rd water	3·0	4·8
4th water	0·8	2·1
Total removed by water	60·2	62·8
Total P extracted	60·2	68·5
Washed with water and TCA		
Extract:		
Lipid solvent	—	5·7
1st water	49·5	41·7
5% TCA	18·7	12·5
2nd water	5·1	11·2
3rd water	4·0	11·0
Total removed by water and TCA	77·3	76·4
Total P extracted	77·3	82·1

Table 6. *Effect of incubation of* pH 9-*insoluble residues on liberation of dialysable phosphorus*

(Sap from grass (mixed *Festuca* spp.) (1 l., 619 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (99·7 mg. P) was treated with lipid solvents and then extracted twice at pH 9. The residue (14·7 mg. P) was suspended in water and divided into two equal portions. One was adjusted to pH 7 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The other portion was taken to pH 9 and dialysed without previous incubation. The inorganic and total P contents of the dialysates are recorded as % of the total initial P of the residue.)

	Fractions as % of total initial P	
	Incubated at 37°	Not incubated
Inorganic P	32·4	13·0
Total P	51·6	38·3

taken to pH 9 and dialysed at room temperature against NaOH, pH 9 (1·5 vol., changed every 12 hr.), till the dialysates contained less than 1–2 μg. P/ml. Control samples were brought to pH 9 immediately and dialysed in the same way. In each case, the dialysates were examined for total and inorganic P content. Incubated samples always showed marked increases over the un-incubated controls, in both inorganic and total P contents of the dialysates. The figures in Table 6 refer to lipid-free samples. Preparations from lipid-containing sap-TCA precipitates showed the same thing.

The P contained in extracts from incubated material is not solely in the form of mononucleotides. This was shown by the fact that if a sample were incubated for 24 hr. and then dialysed at pH 9 until no further P passed through the membrane, about 90% of the P of the residual material (still at pH 9) was in solution. About half of it separated with the proteins when they were precipitated from the dialysed solutions by addition of $(NH_4)_2SO_4$ to 40% saturation.

The above results clearly indicate retention by the insoluble residues of small amounts of the enzyme activity of the original sap. This was confirmed by analysis of the purine and pyrimidine contents of the incubation extracts, and by examination of the sap-TCA precipitates for presence of enzyme activity.

did not liberate further P on incubation. The extracts obtained after boiling contained less P than did those after incubation, and the ratio of purines to pyrimidines becoming soluble was greater (Table 7). As in the case of the incubated samples, more adenine was obtained when the residues were treated at 100° for 30 min. with 5% (w/v) TCA, but even so, about 10% of the initial pH 9-insoluble P still remained insoluble after these treatments. The experiment whose results are presented in Table 7, was repeated a further four times with similar results.

It will be seen that there was no significant difference in either the total amount of P extracted or in the purine and pyrimidine composition of the combined extracts, whichever of these two series of extractions of pH 9-insoluble residues was followed. However, enzyme activity occurring during incubation at 37° leads to extracts containing pyrimidines, most of which are not liberated when enzymic activity is destroyed by boiling the samples.

Phosphorus associated with protein precipitates

The figures in Table 1 show that a large proportion of the P in the pH 9 extracts of the sap-TCA precipitates separated together with the proteins when these were precipitated from the extracts. As in the case of the pH 9-insoluble residues, this appears to exist in polynucleotide combination. If the precipitates were redissolved in NaOH, pH 9, immediately after precipitation and kept at 4° for several hours before re-precipitation, only an insignificant fraction (about 5%) of this P failed to reprecipitate.

The precipitates displayed enzymic activity. Samples were incubated at 37° in water at pH 7 for 24 hr., taken to pH 9 and dialysed against NaOH, pH 9 (1·5 vol., changed every 12 hr.), at room temperature, till the dialysates contained less than 1–2 μg. P/ml. The dialysates showed marked increases in both total and inorganic P contents over those from control samples which were dialysed at pH 9 without prior incubation. The effect was shown by both lipid-containing and lipid-free samples of proteins from sap-TCA precipitates.

Table 7. *Purine and pyrimidine composition of the phosphorus fractions obtained from* pH 9*-insoluble residues by various treatments*

(Sap (1 l., 0·213 g. P) was converted to the sap-TCA precipitate (0·047 g. P). This was treated with lipid solvents and then extracted twice at pH 9. The residue (10·34 mg. P) was suspended in water and split into two equal portions. One portion was incubated at 37° for 24 hr. at pH 7. After centrifuging down, the residue was washed twice with water on the centrifuge, treated with 5% (w/v) TCA at 100° for 30 min., centrifuged down and washed with water again. The combined extracts contained 4·63 mg. P. The other portion was treated in exactly the same way except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 4·79 mg. P in the combined extracts. The purine and pyrimidine content of the extracts is given as molecules base/100 atoms P in the combined extracts.)

	Extract	Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	1·6	8·1	57·9	65·2
	Water (2)	0·8	2·0	3·8	6·9
	5% (w/v) TCA	6·2	3·6	11·7	21·4
	Water	1·8	1·3	4·3	6·5
	Total	10·4	15·0	77·7	100
Boiled 5 min.	Boiled	1·4	10·7	24·8	40·0
	Water (2)	1·0	—	5·3	6·6
	5% (w/v) TCA	5·7	2·3	34·4	42·7
	Water	1·4	—	8·7	10·7
	Total	9·5	13·0	73·2	100

Nature of phosphorus fractions liberated on incubation of pH 9*-insoluble residues*

Incubation of lipid-free pH 9-insoluble residues led to extracts containing predominantly pyrimidines (Table 7). The proportion of adenine in the samples, however, was much greater than in the nucleotide preparations. Further amounts of adenine were liberated when the residues after incubation were heated in 5% (w/v) TCA at 100° for 30 min. About 10% of the P present before incubation remained with the insoluble residues after these treatments.

The enzyme activity of the samples was destroyed by boiling for 5 min. After such treatment the preparations

About 90% of the P remaining non-dialysable after incubation was soluble at pH 9. If the proteins in these extracts were thrown out by 40% saturation with $(NH_4)_2SO_4$, 50–70% of the P was co-precipitated.

Table 8. *Effect of incubation of protein precipitates upon liberation of dialysable phosphorus*

(Sap (1 l., 212 mg. P) was treated with TCA. The sap-TCA precipitate (29·6 mg. P) was split into two equal portions. One portion was fractionated at pH 9 without removal of lipids. The other was extracted with lipid solvents prior to fractionation. The protein precipitates separating from the alkaline extracts of the sap-TCA precipitates at pH 6, contained 1·04 mg. P and 1·68 mg. P respectively. Each precipitate was then split into two equal portions. One portion was, in each case, incubated at 37° in water at pH 6 for 24 hr., taken to pH 9 and dialysed as described in the text: the remaining portions were dialysed at pH 9 without previous incubation. The inorganic and total P contents of the dialysates are recorded in the table as % of the total initial P of the protein precipitates.)

	Lipids present		Lipids absent	
	Incubated	Not incubated	Incubated	Not incubated
Inorganic P	18·5	6·0	17·7	9·8
Total P	82·7	63·5	80·6	54·0

Effect of incubation of protein precipitates on nature of phosphorus fractions liberated

Extracts obtained by incubating the protein precipitates at pH 6 for 24 hr. contained an excess of purines over pyrimidines (Table 9). Further quantities of nucleotides, also containing more purines than pyrimidines, were released when the residues were heated at 100° for 30 min. in 5% (w/v) TCA. About 10% of the P initially present failed to become soluble following these treatments.

Boiling a suspension of the precipitates at pH 6 for 5 min. destroyed the enzymic activity. The boiled extracts contained rather less P than did the incubation extracts, but as was found with incubated samples, purines predominated over pyrimidines in the extracts. Treatment of the residues with 5% (w/v) TCA at 100° liberated further quantities of P, and a total of about 90% of the P initially present could be extracted from the protein precipitates by these combined treatments. Table 9 shows that approximately the same total amounts of P, of purines and of pyrimidines, are liberated, whichever set of extraction conditions are employed. However, enzymic activity during incubation of the protein precipitates gives extracts containing more nitrogen bases (especially purines) than does boiling the samples, which destroys the enzymes. Fractionation of the P of the protein precipitates by the above methods gave similar results on several more samples.

Fractionation of chloroplast-TCA precipitate

Preparation and properties of mononucleotide solutions

Chloroplast-TCA precipitate was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless. The lipid-free residue was then put through the same alkaline fractionation procedure as was used for the preparation of nucleotides from sap-TCA precipitates.

The amounts of P obtained in various fractions in this way, are given in Table 10. These figures refer to chloroplasts obtained from the sap preparations whose P distribution on fractionation is detailed in Table 1. The figures in these two tables differ in a number of respects. Sap-TCA precipitates contain smaller amounts of P extractable by lipid solvents (5–10% of the total) than do chloroplast-TCA precipitates. Two lipid-containing chloroplast-TCA precipitates retained only about 30% of their P after two extractions at pH 9, instead of the 40–55% remaining in samples previously treated with lipid solvents. However,

Table 9. *Purine and pyrimidine composition of the phosphorus fractions obtained from protein precipitates by various treatments*

(Sap (1 l., 96·1 mg. P) was converted to the sap-TCA precipitate (37·8 mg. P). On treatment with lipid solvents followed by fractionation, as described in the text, this yielded a protein precipitate at pH 5 containing 3·8 mg. P. This precipitate was suspended in water and split into equal portions. One portion was incubated at 37° for 24 hr. at pH 6. After centrifuging down, the residue was washed twice with water, heated in 5% (w/v) TCA at 100° for 30 min., again centrifuged down and washed twice more with water. The extracts contained a total of 0·84 mg. P. The second portion was treated in exactly the same way, except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 0·82 mg. P in the combined extracts. The purine and pyrimidine contents of the various extracts is given as molecules base/100 atoms P in the combined extracts.)

	Extract	Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	15·7	45·1	6·5	69·5
	Water (2)	1·2	—	—	1·3
	5% (w/v) TCA	4·9	18·4	3·3	27·1
	Water	2·1	—	—	2·1
	Total extracted	23·9	63·5	9·8	100
Boiled 5 min.	Boiled	11·9	37·5	3·4	52·2
	Water (2)	1·8	—	1·7	3·4
	5% (w/v) TCA	7·4	26·7	7·4	40·9
	Water (2)	2·2	1·1	—	3·5
	Total extracted	23·3	65·3	12·5	100

even when allowance is made for the decreased extent of extraction following removal of lipids, there is still a much smaller percentage P extraction from chloroplast-TCA precipitates than from sap-TCA precipitates. Again, only a small proportion of the P in the pH 9 extracts could be isolated in mononucleotide preparations. About half of this pH 9-soluble P is precipitated, together with the proteins, from the extracts on acidification, but its nature has not been investigated further.

Incubation of the pH 9-insoluble residues at pH 6 led to increases, over non-incubated controls, in the amount of P that could be subsequently dialysed away from the preparations at pH 9, but these increases were small.

Incubation of the pH 9-insoluble residues at 37° for 24 hr. with a sap ribonuclease preparation of the type described by Pirie (1950) (used in preference to that described in the next section because of its low dry matter and P content) in amounts more than sufficient to cause practically complete fission in 24 hr. of a yeast nucleic acid solution (pH 6) with the same total P content as the chloroplast residues, brought out only some 50–60% of the P. Most of the remainder was released when incubation was repeated twice more with fresh enzyme solution. The remaining P (approx. 8% of the initial total) was not brought out by further incubations, or by cold or hot 5% (w/v) TCA treatment.

Table 10. *Distribution of phosphorus in some fractions from chloroplasts*

(The values given are the extreme ranges obtained with several different batches of chloroplasts.)

	Percentage of total P of the		
	Chloroplasts	Chloroplast-TCA precipitate	pH 9 extracts
Soluble in lipid solvents	15–20	20–30	—
pH 9-insoluble residues	25–40	40–55	—
Mononucleotide preparations	2–8	4–10	15–30
Protein precipitates	10–15	15–20	45–55

Nucleotide preparations and protein precipitates account in most cases for about 70% of the alkali-soluble P. About half of the remainder is found in the aqueous ethanolic supernatants from the nucleotide precipitates. The nature of this baryta-soluble, ethanol-soluble P has not been investigated. Small amounts of P are also found associated with the $BaSO_4$ precipitates, and in the precipitates separating at pH 9 before addition of ethanol. It is thus usually possible to account for over 90% of the P of the chloroplast-TCA precipitate after fractionation.

The nucleotide preparations had the same general properties as did those from the sap-TCA precipitates. Analyses of typical preparations are given in Table 11, which shows that, as in the case of sap preparations, pyrimidines predominate over purines.

Table 11. *Purine and pyrimidine composition of chloroplast nucleotide preparations*

(Results of two typical experiments are presented as molecules base/100 atoms P. The chloroplasts, containing 100 mg. P in each case, gave in exp. 1 a TCA precipitate (83·1 mg. P) and nucleotide preparation (8·03 mg. P), and in exp. 2 a TCA precipitate (71·9 mg. P) and nucleotide preparation (4·03 mg. P.)

Preparation	Adenine	Guanine	Total pyrimidines
	(molecules/100 atoms P)		
1	2·1	32·1	63·6
2	0·8	34·7	61·6

Phosphorus content of pH 9-*insoluble material of chloroplast-TCA precipitate*

This is a considerable fraction of the initial chloroplast-TCA precipitate total (Table 10). If these preparations were incubated at 37° for 24 hr. in water at pH 6, 30–40% of the P was extracted. Repeated incubations at pH 6, the water being replaced every 24 hr., eventually led to extraction of about 70% of the P. Treatment of the residual material with 5% (w/v) TCA brought out a further 5% at room temperatures, and 15–20% after 30 min. at 100°.

Table 12. *Effect of incubation upon liberation of dialysable phosphorus from residues of chloroplast-TCA precipitate insoluble at* pH 9

(Washed chloroplasts were suspended in water and precipitated with TCA. The precipitate was treated several times with lipid solvents and then extracted twice at pH 9. The residue was suspended in water and split into two equal portions. One portion was adjusted to pH 6 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The remaining portion was dialysed at pH 9 without previous incubation. In exp. 1, chloroplasts (100 mg. P) gave a TCA precipitate (52·09 mg. P) and nucleotide preparation containing 2·79 mg. P. In exp. 2, the chloroplasts (100 mg. P) gave 2·78 mg. P in nucleotide preparation from a TCA precipitate containing 56·89 mg. P. The inorganic and total P contents of the dialysates are recorded as the percentage of the total initial P of the residues.)

	Incubated		Not incubated	
Experiment ...	1	2	1	2
Inorganic P	2·1	3·9	—	—
Total P	11·6	13·2	6·1	3·0

Very little P was extracted by overnight treatment of the pH 9-insoluble residues with N-$HClO_4$ at room temperatures, but incubation at 37° for 24 hr. followed by washing with water brought out over 80% of the phosphorus.

The P in extracts obtained from the pH 9-insoluble residues following incubation in water (with and without added ribonuclease), or in N-$HClO_4$, exhibited strong ab-

sorption in the region of 260 mμ. Paper chromatography of samples after hydrolysis in N-HCl for 60 min. showed the presence of adenine, guanine, cytidylic and uridylic acids. No figures have been obtained for the relative proportions of the various nitrogen bases in these extracts.

Effect of TCA precipitation upon activity of sap ribonuclease and phosphatase

Sap was split into equal halves. One lot was dialysed against distilled water at 4°, with frequent changes, for 3 days, and the volume noted. The other was precipitated with TCA and the sap-TCA precipitate, after a preliminary water wash, was suspended in water and dialysed in the same way. It was then suspended in water and made up to a known volume.

Both sap and sap-TCA precipitate preparations exhibited ribonuclease activity, with optimum fission occurring at about pH 5·5. The activity of the sap-TCA precipitate preparations was much lower than that of the sap. For example, 30% fission of a yeast nucleic acid solution was brought about by 0·125 ml. dialysed sap (equivalent to 0·107 ml. sap before dialysis) in 2·75 hr. It needed 1·5 ml. of the corresponding sap-TCA precipitate preparation (from 1·8 ml. sap before precipitation and dialysis) to give this same fission in 4·5 hr. Hence the sap-TCA precipitates still exhibited about 4% of the activity of an equivalent volume of sap.

Dialysed sap exhibited phosphatase activity towards a number of substrates. This diminished on precipitation with TCA to a much greater extent than did the ribonuclease activity. Thus, while a sample of 0·05 ml. dialysed sap (equivalent to 0·04 ml. sap before dialysis) split sodium β-glycerophosphate solution to the extent of 21% in 2 hr. at 37°, 1·5 ml. of the corresponding sap-TCA precipitate (equivalent to 1·2 ml. original sap) only caused 3% fission in 72 hr. at 37°. Hence only about 1 part in 7500 of the original phosphatase activity remained after TCA precipitation. The use of metaphosphate and yeast adenylic acid as substrates gave similar results.

DISCUSSION

Kaufmann, Gay & McDonald (1951) have shown that pancreatic ribonuclease attacks pancreatic nucleoprotein as well as the free nucleic acid. The enzyme is stable to extremes of pH, and displays marked activity at pH 9·5 (Dubos & Thompson, 1938), although it is largely inactivated in 5 min. at pH 7 and 100° (Kunitz, 1940). It would seem from the present results that the reactions leading to the formation of nucleotides from sap-TCA precipitates are at least partially enzymic in character. The results are compatible with the theory that the ribonucleoprotein in the sap-TCA precipitate is being degraded to varying extents by the sap ribonuclease. The preferential fission of pyrimidine-containing fragments from yeast nucleic acid by means of pancreatic ribonuclease has been noted by a number of workers, and has also been demonstrated for the ribonucleic acids of tobacco mosaic and turnip yellow viruses and of rabbit liver (Markham & Smith, 1951). Carter & Cohn (1950) showed that the acid-soluble digestion products produced from yeast nucleic acid by ribonuclease consisted almost exclusively of pyrimidine nucleotides, and suggested that the very small amounts of adenylic and guanylic acids also isolated were artifacts obtained during the isolation procedure from material of higher molecular weight.

The presence of purines in the nucleotide mixtures obtained here from sap may be due to purely chemical degradation of the nucleic acid (cf. Schmidt & Thannhauser, 1945). However, Pirie (1950) has already noted that sap ribonuclease differs from the pancreatic enzyme in that it brings about more or less complete fission of yeast and sap ribonucleic acids, so that the products are no longer capable of precipitation by the uranyl reagent. Moreover, Kunitz (1940) reported an optimum pH of 7·7 for pancreatic ribonuclease activity, whereas the sap enzyme has been found to exhibit maximum activity at pH 5–6. It is, therefore, possible that the presence of purines in the sap preparations is due to ribonuclease activity, and this is supported by the exhibition of enzymic activity, leading to the almost exclusive liberation of purine nucleotides, by the protein precipitates obtained during isolation of the nucleotide preparations. The low yield of nucleotides obtained when pH 9-insoluble material and protein precipitates were pooled and again fractionated also agrees with enzymic, as opposed to purely chemical, decomposition during alkaline fractionation.

Bacher & Allen (1950) recently showed that the usual methods of preparation of pancreatic ribonucleic acid yielded products containing ribonuclease. Pirie (1950) described the similar association of ribonuclease activity with tobacco sap nucleoprotein. He also showed the partial breakdown of the sap nucleic acid when prepared from the nucleoprotein by decomposition with TCA, even fresh preparations yielding a maximum of only 60% of the phosphorus in the nucleic acid. Starting with sap, he was unable to obtain any undegraded nucleic acid by precipitation with TCA, and he ascribed this partly to decomposition on prolonged contact of the nucleoprotein with TCA, and partly to enzyme action in the sap or during the preparation. The present results are in agreement with these conclusions. Enzyme activity in the sap-TCA precipitate and in various fractions obtained during the fractionation procedure, has been amply demonstrated. The evidence concerning the effect of contact with TCA on the sap-TCA precipitate, although it is not unequivocal, as the results were somewhat variable, also supports the above conclusions. Most of the present work has been done on sap-TCA precipitates collected immediately after preparation, but a small number of experiments

were performed in which these precipitates were allowed to age in contact with the TCA supernatant before being collected. In general, rather more phosphorus was found in a precipitate collected immediately after preparation than in an aged precipitate, the difference being greater the longer the precipitate was aged. It appears that sap nucleic acid is more sensitive to decomposition by TCA than are other nucleic acids (cf. Schneider, 1945). Ageing a sap-TCA precipitate decreased the solubility of the sap proteins at pH 9, but the part that went into solution retained on reprecipitation an amount of phosphorus larger than that separating with the proteins from a fresh sap-TCA precipitate. This is probably due to preferential absorption of nucleic acid or polynucleotide material by the denatured proteins.

The formation of nucleotides from chloroplast-TCA precipitates probably follows the same pattern as for sap. However, the smaller proportion of material soluble at pH 9, and of nucleotides isolable from this material, suggests either that the ribonucleic acid of chloroplasts is present in two fractions, one of which is not readily accessible to ribonuclease action, or that chloroplasts contain a ribonuclease inhibitor. Repeated additions of large amounts of sap ribonuclease were found to be necessary to extract the remaining nucleic acid from chloroplast residues, so that the low extent of extraction from the initial TCA precipitate and the slow rate of liberation of phosphorus from the pH 9-insoluble residues, do not appear to be due to enzyme inhibition. Moreover, it has recently been found (Holden, 1951) that only about 40% of the chloroplast ribonucleic acid is extracted by N-$HClO_4$ at room temperature, incubation at 37° being necessary to remove the remainder. Ogur & Rosen (1950) claimed complete extraction of ribonucleic acid from plant tissues under the former conditions.

The existence of phosphatase activity in the pH 9-insoluble residues and the protein precipitates is rather surprising in view of the low activities found for the sap-TCA precipitates. It is possible that traces of the enzyme escape denaturation during TCA precipitation, through being carried down mechanically in the precipitate, and are subsequently released when the precipitates are brought to pH 9.

It was noticed during incubation of pH 9-insoluble residues, suspensions of protein precipitates and of sap-TCA precipitates, that the suspensions rapidly settled and changed gradually, over a period of several hours, to a harder, more compact form. This did not affect the enzymic activity of the samples, since agitation did not enhance it, although it delayed completion of these changes.

SUMMARY

1. A fractionation procedure for following the breakdown of sap and chloroplast ribonucleic acids at pH 9, following precipitation with trichloroacetic acid, is described.

2. Both sap and chloroplast nucleic acids were partially degraded to a mixture of mononucleotides containing an excess of pyrimidines over purines.

3. The remaining breakdown products were found to be contained, in polynucleotide combination, principally in material insoluble at pH 9 and in protein precipitates obtained during fractionation. The purine and pyrimidine distribution following enzymic and chemical degradation of these fractions is described.

4. Differences in the ease of degradation of sap and chloroplast nucleic acids are described.

5. The extent of denaturation of sap ribonuclease and phosphatase following precipitation with trichloroacetic acid was determined.

I wish to thank the Agricultural Research Council for a grant to make this work possible.

REFERENCES

Bacher, J. E. & Allen, F. W. (1950). *J. biol. Chem.* **183**, 641.

Buell, M. V. (1943). *J. biol. Chem.* **150**, 389.

Carter, C. E. & Cohn, W. E. (1950). *J. Amer. chem. Soc.* **72**, 2604.

Daly, M. M., Allfrey, V. G. & Mirsky, A. E. (1950). *J. gen. Physiol.* **33**, 497.

Dubos, R. J. & Thompson, R. H. S. (1938). *J. biol. Chem.* **124**, 501.

Euler, H. von & Hahn, L. (1947). *Ark. Kemi Min. Geol.* **25B**, no. 1.

Euler, H. von & Hahn, L. (1948). *Ark. Kemi Min. Geol.* **26A**, no. 11.

Foreman, F. W. (1938). *J. agric. Sci.* **28**, 135.

Holden, E. M. (1951). (Personal communication.)

Holiday, E. R. & Johnson, E. A. (1949). *Nature, Lond.*, **163**, 216.

Kaufmann, B. P., Gay, H. & McDonald, M. R. (1951). *Amer. J. Bot.* **38**, 268.

Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.

Kuttner, T. & Lichtenstein, L. (1932). *J. biol. Chem.* **95**, 661.

McFadyen, D. A. (1934). *J. biol. Chem.* **107**, 299.

Markham, R. & Smith, J. D. (1951). *Research*, **4**, 344.

Markham, R. & Smith, K. M. (1949). *Parasitology*, **39**, 330.

Michaelis, L. (1931). *Biochem. Z.* **234**, 139.

Militzer, W. E. (1946). *Arch. Biochem.* **9**, 85.

Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.

Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 269.

Pirie, N. W. (1950). *Biochem. J.* **47**, 614.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
Soodak, M., Pircio, A. & Cerecedo, L. R. (1949). *J. biol. Chem.* **181**, 713.
Takasugi, N. (1943). *J. chem. Soc. Japan*, **64**, 869, 1375.
Takasugi, N. (1944). *J. chem. Soc. Japan*, **65**, 333.
Vischer, E. & Chargaff, E. (1948*a*). *J. biol. Chem.* **176**, 703.
Vischer, E. & Chargaff, E. (1948*b*). *J. biol. Chem.* **176**, 715.
Williams, J. N. (1950). *J. biol. Chem.* **184**, 627.
Woodhouse, D. L. (1950). *Arch. Biochem.* **25**, 347.
Zittle, C. A. (1946). *J. biol. Chem.* **163**, 119.

Amine Oxidase Activity of Rat Liver in Riboflavin Deficiency

By JOYCE HAWKINS
*Department of Pharmacology, Oxford**

(*Received* 12 *October* 1951)

The chemical constitution of the enzyme amine oxidase is not known. There are some indications that the closely related enzyme histaminase, which also catalyses the oxidation of amines, is a flavoprotein (Zeller, Stern & Wenk, 1940; Kapeller-Adler, 1949). It is known that D-amino-acid oxidase contains riboflavin in the form of flavin-adenine dinucleotide (FAD) (Warburg & Christian, 1938). In riboflavin deficiency there occurs a marked reduction in the D-amino-acid oxidase activity of rat liver (Axelrod, Soker & Elvehjem, 1939, 1940; Rossiter, 1940), and the FAD content of the liver is reduced (Ochoa & Rossiter, 1939).

In the present work the effect of riboflavin deficiency on the amine oxidase activity of rat liver has been studied. The D-amino-acid oxidase activity has been determined at the same time in order that this might serve as an index of the degree of the riboflavin deficiency obtained. In this study characteristic differences in the two enzymes have been observed.

METHODS

Diet. The method adopted for inducing riboflavin deficiency in rats was based on that described by Carpenter Harris & Kodicek (1948) who used a diet low in protein and containing succinylsulphathiazole. I am grateful to Dr E. Kodicek for his advice on the use of this diet.

The diet was composed as follows: riboflavin-free casein 10·5%, sucrose 80·35%, salt mixture (Steenbock no. 40) 5%, L-cystine 0·15%, arachis oil 3%, and succinylsulphathiazole 1%. This diet was fed to the rats *ad lib.* As supplements, they received daily: 30 μg. aneurin, 30 μg. pyridoxine, 20 mg. choline chloride, 1 μg. biotin, 250 μg. nicotinic acid and 200 μg. calcium pantothenate; three times weekly they were given 40 μg. folic acid dissolved in 1% KH_2PO_4, and once a week one drop of a 5% solution of tocopherol acetate, four drops of cod-liver oil and 1 mg. menaphthone. The animals which received this diet without added riboflavin are called deficient rats. The animals which received a daily supplement of 30 μg. riboflavin are called controls. Modifications of the diet, as used in some of the experiments, will be described later.

Animals. Litters of freshly weaned male and female piebald rats, obtained from Glaxo Laboratories, were used. They weighed about 30 g. on arrival.

About two-thirds of the rats in each litter received the deficient diet. In most litters the remaining animals were kept as controls and received daily supplements of riboflavin.

Tissue preparations. Each liver was homogenized in a small volume of 0·067 M-sodium phosphate buffer, pH 7·4. More buffer was added so that 5·0 ml. homogenate contained 1 g. tissue. About one-half of the homogenate was retained for amine oxidase determination, the remainder was centrifuged at 600*g* for 5 min. and the supernatant used for D-amino-acid oxidase determination.

Kidney extracts were prepared by first grinding the tissue with sand in a mortar, and then adding 0·067 M-sodium phosphate buffer, pH 7·4, so that 5·0 ml. suspension contained 1 g. tissue. This suspension was centrifuged for 5 min. at 600*g* and the supernatant was used for D-amino-acid oxidase determination.

Measurement of enzymic activity. All determinations were made at 37° and in O_2. Conical manometer flasks were used; the inner compartments contained filter paper and 0·3 ml. N-KOH.

Amine oxidase activity. This was determined with tyramine as substrate. The side bulbs contained either 0·4 ml. of 0·05 M-tyramine hydrochloride, or 0·4 ml. of water in the blank. The main compartment of each flask contained 1·0 ml. of rat-liver homogenate plus 0·2 ml. of 0·1 M-semicarbazide plus 0·4 ml. of water, to trap the aldehyde formed during the initial oxidation of tyramine in order to prevent further oxidation.

For the study of D-amino-acid oxidase, DL-proline was chosen, as D-proline was found to be oxidized at a high rate by the rat enzyme. The side bulb contained 0·5 ml. 1·0 M-DL-proline; the main compartment contained 0·5 ml. liver extract plus 0·5 ml. water. In the experiments with kidney the proline concentration was 0·1 M.

* Present address: Department of Chemistry and Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1.