5. *The Constitution of Yeast Ribonucleic Acid. Part X. Further Studies on the Nature of the Carbohydrate Radicals.*

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The carbohydrate radicals of the purine nucleotides of yeast pentose nucleic acid are shown to be *d*(−)-ribose by identification of the corresponding aldonic acids as benziminazoles. Results previously reported (*J.*, 1943, 625; 1944, 339), suggesting that some *d*-arabinose might also be present, are now proved to have been due to epimerisation during the condensation of *d*-ribonic acid with *o*-phenylenediamine; the course of the reaction is dependent on the concentration of hydrogen chloride, and leads to ribobenziminazole alone when this is sufficiently high.

An examination of the literature having revealed inconsistencies in the designations of the carbohydrate radicals of the pentose nucleic acids, attempts were made (Barker and Gulland, *J.*, 1943, 625) to establish the nature of the sugar of the pentose nucleic acid of yeast by formation of benziminazoles (Moore and Link, *J. Biol. Chem.*, 1940, **133**, 293) from the corresponding aldonic acids. Since the same benziminazole was obtained from synthetic barium *d*-ribonate (Steiger, *Helv. Chim. Acta*, 1936, **19**, 189) and from the aldonic acids produced by oxidation of the carbohydrate radicals liberated during acid hydrolysis of yeast pentose nucleic acid and the guanylic, adenylic, and cytidylic acids derived from it, it was concluded that these sugar radicals were *d*(−)-ribose.

After that investigation had been completed, however, the properties of *d*-ribobenziminazole

were reported both by Dimler and Link (*J. Biol. Chem.*, **1943**, **150**, 345), and by Richtmyer and Hudson (*J. Amer. Chem. Soc.*, **1942**, **64**, 1612) as being completely different from those found by us. The behaviour of *d*-ribonic acid was therefore re-examined, and *d*-ribobenziminazole was obtained (Barker, Cooke, and Gulland, *J.*, **1944**, **339**), having properties corresponding with those observed by the American authors. The properties of the compound previously obtained by us were the same as those of *d*-arabobenziminazole, and the identity was confirmed by direct comparison with a sample prepared from synthetic *d*-arabonic acid obtained by oxidation of *d*(−)-arabinose; the melting point of our *d*-arabobenziminazole is, however, rather higher than that given by Moore and Link (*loc. cit.*). It seemed, therefore, that yeast pentose nucleic acid might contain *d*(−)-arabinose. The present investigations, limited to the sugar radicals associated with the purine bases in the nucleic acid, were designed to test this point.

By using the method of isolating *d*-ribobenziminazole recommended by Dimler and Link (*loc. cit.*, and private communication), it was found that our failure to isolate this compound in previous experiments was due to its high solubility, particularly in the presence of inorganic salts. Nevertheless, the aldonic acid obtained from guanosine still yielded *d*-arabobenziminazole as before in some experiments, in addition to *d*-ribobenziminazole. Furthermore, since this result was obtained using apparently identical samples of guanosine prepared from yeast nucleic acid supplied by both British Drug Houses and The Pharmaco-Chemical Products Co., it seemed likely that the formation of two products was to be ascribed not to the composition of any particular specimen of nucleic acid but rather to the mechanisms of the breakdown of the molecule or of the production of the benziminazole. Subsequent experiments confirmed this view since either *d*-ribobenziminazole or a mixture of this and *d*-arabobenziminazole could be produced at will. They also confirmed that these two commercial specimens of yeast nucleic acid were identical as regards the carbohydrate radicals of their purine nucleotides.

Dimler and Link (*loc. cit.*) point out that if oxidation of *d*(−)-ribose is carried out with alkaline hypoiodite, epimerisation results in the formation of some *d*-arabobenziminazole. This could, no doubt, explain the isolation by us of *d*-arabobenziminazole from yeast nucleic acid as previously reported, since oxidation was then carried out with alkaline hypoiodite, but in the experiments with nucleotides hydrolysis and oxidation were achieved simultaneously with hydrogen bromide and bromine. In the present investigations in which *d*-ribobenziminazole alone was formed, sugars were oxidised by bromine in the presence of barium benzoate (Hudson and Isbell, *J. Res. Nat. Bur. Stand.*, **1929**, **3**, 57), and parallel experiments using simultaneous hydrolysis and oxidation gave identical results. It was therefore concluded that *d*-arabonic acid was not formed during oxidation in acid solution. The possibility that *d*-ribobenziminazole suffered epimerisation in the feebly alkaline solution used in its preparation (see Experimental section) was excluded by the failure of deliberate attempts to effect this change.

It was also found that the nature of the benziminazoles produced was not dependent on the method used for disrupting the molecule of nucleic acid, since the same results were obtained whether it was broken down initially into nucleotides by cold alkali, or into nucleosides by hot aqueous pyridine, or by refluxing with acid whereby the sugar is liberated directly.

It was observed, however, that, quite independently of the technique used for hydrolysis and oxidation, either ribobenziminazole alone or ribobenziminazole together with arabobenziminazole could be produced by varying the quantity of hydrogen chloride used in the condensation of the aldonic acid with *o*-phenylenediamine. Thus one and the same sample of calcium aldonate obtained from guanosine, when condensed in presence of 2·48 molecular equivalents of hydrogen chloride yielded only *d*-ribobenziminazole, whereas in the presence of 1·4 molecular equivalents both ribobenziminazole and arabobenziminazole were isolated. In a parallel experiment with a different sample of calcium aldonate both benziminazoles were obtained using 1·98 molecular equivalents. It is therefore concluded that variations in the conditions of condensation alone account for the anomalous results and that, providing they are satisfactorily controlled, only *d*-ribobenziminazole is obtained from *d*-ribonic acid. The decision that the use of the higher, and not the lower, concentration of hydrogen chloride gave the correct result is justified by the formation of *d*-ribonic phenylhydrazide alone, but not *d*-arabonic phenylhydrazide, from ribonic acid which yielded ribobenziminazole either alone or mixed with arabobenziminazole according to the conditions of condensation with *o*-phenylenediamine (*J.*, **1943**, **625**; **1944**, **339**). Levene's conclusion that *d*(−)-ribose is the carbohydrate constituent of the purine nucleotides of yeast pentose nucleic acid is thus confirmed.

Care is required in the application of the benziminazole technique to the identification of sugars. Thus, consideration of the conditions recommended by Dimler and Link (*loc. cit.*) for preparing *d*-ribobenziminazole indicates that the formation of arabobenziminazole, which did

not occur, should not have been expected. On the other hand, repeated attempts have so far failed to yield *l*-ribobenziminazole in the condensation of calcium arabonate [prepared from *l*(+)-arabinose] with *o*-phenylenediamine in presence of hydrogen chloride in an amount insufficient to prevent epimerisation of calcium ribonate. Moore and Link (*J. Org. Chem.*, 1940, **5**, 637) state that in absence of hydrogen chloride epimerisation occurs at 135—150°; they isolated lyxobenziminazole from the condensation of *o*-phenylenediamine and xylonic acid, and add that this epimerisation is prevented by acid catalysts. In some of the condensations described in our two previous papers, phosphoric acid, as well as hydrogen chloride, was used to aid interaction, but had no effect on the course of the reaction which seemed to be guided by the concentration of hydrogen chloride. Determination of the chloride ions in a mixture of *o*-phenylenediamine and calcium arabonate after it had been condensed indicated a considerable loss of hydrogen chloride during the reaction.

Experimental.

Benziminazoles from Guanosine.—(i) Guanosine (15 g.), prepared from yeast nucleic acid (The Pharmaco-Chemical Products Co. Ltd.) as described by Bredereck, Martini, and Richter (*Ber.*, 1941, **74**, 694), was heated under reflux for 2 hours with N/10-sulphuric acid (2·5 l.). Silver sulphate (20 g.) was added to the hot solution, and next day the precipitate was removed and the solution was freed from silver ions by passage of hydrogen sulphide, filtration, and aeration, and from sulphate ions by addition of hot saturated baryta and filtration from barium sulphate. Analysis (Hinton and Macara, *Analyst*, 1924, **49**, 2) showed the solution to contain 6·45 g. of pentose. After concentration of the solution under reduced pressure to 350 c.c., the pentose was oxidised as described by Hudson and Isbell (*loc. cit.*), and the resulting solution of aldonic acid was refluxed for 3 hours with calcium carbonate. Excess of calcium carbonate was removed, and the filtrate was concentrated under reduced pressure to 20 c.c. and added to 95% ethyl alcohol (350 c.c.). After thorough cooling, the precipitate (7·8 g.) was collected and dried. This calcium aldonate (4 g.) was heated at 130—140° for 2 hours with *o*-phenylenediamine (1·58 mols.), concentrated hydrochloric acid (1·98 mols. of hydrogen chloride), water (8 c.c.), and ethyl alcohol (2 c.c.). The resulting syrup was dissolved in hot water (50 c.c.), decolorised with charcoal, and made alkaline to litmus by addition of 4N-sodium hydroxide (6 c.c.) and a few drops of ammonia (*d* 0·880). After being cooled in the refrigerator, the precipitated arabobenziminazole was collected, and when crystallised from hot water formed needles, m. p. 240—241° not depressed by admixture with synthetic *d*-arabobenziminazole (Found : N, 11·7. Calc. for $C_{11}H_{14}O_4N_2$: N, 11·8%). In 5% aqueous citric acid (*c*, 2·0) $[\alpha]_D^{20°}$ was − 49·5°. Richtmyer and Hudson (*loc. cit.*) give $[\alpha]_D^{20°}$ − 49·4°; Moore and Link (*loc. cit.*) give $[\alpha]_D^{25°}$ − 51°. The picrate had m. p. 158—159° (Found : N, 14·5. Calc. for $C_{11}H_{14}O_4N_2,C_6H_3O_7N_3$: N, 15·0%), and the hydrochloride had m. p. 230—232° (Found : N, 10·2. Calc. for $C_{11}H_{14}O_4N_2,HCl$: N, 10·2%); Moore and Link (*loc. cit.*) give m. p. 158° and 229° respectively.

The filtrate from *d*-arabobenziminazole, after removal of *o*-phenylenediamine by extraction with ether, was evaporated to dryness under reduced pressure. Water (10 c.c.) was added and, after being cooled in ice, the impure *d*-ribobenziminazole was collected and crystallised twice from water from which it separated in needles, m. p. 189—191° not depressed by admixture with synthetic *d*-ribobenziminazole (Found : N, 11·9. Calc. for $C_{11}H_{14}O_4N_2$: N, 11·8%). In 5% aqueous citric acid (*c*, 2·0) $[\alpha]_D^{20°}$ was + 22·0°; Richtmyer and Hudson (*loc. cit.*) give $[\alpha]_D^{20°}$ + 21·6°, and Dimler and Link (*loc. cit.*) give $[\alpha]_D^{20°}$ + 22·5° in N-hydrochloric acid. The picrate had m. p. 184—186° (Found : N, 14·9. Calc. for $C_{11}H_{14}O_4N_2,C_6H_3O_7N_3$: N, 15·0%), and the hydrochloride had m. p. 200—202° (Found : N, 10·2. Calc. for $C_{11}H_{14}O_4N_2,HCl$: N, 10·2%); Dimler and Link (*loc. cit.*) give m. p. 185—186° and 196—198° respectively.

(ii) The previous experiment was repeated exactly using guanosine prepared from yeast nucleic acid supplied by British Drug Houses Ltd. *d*-Arabo- and *d*-ribo-benziminazoles were obtained as before.

(iii) Guanosine from the same sample as that used in experiment (i) was hydrolysed in the same way as before, the sugar solution so obtained was oxidised by the same method, and the calcium aldonate was condensed with *o*-phenylenediamine by the same procedure except that 2·48 mols. of hydrogen chloride were used instead of 1·98. *d*-Ribobenziminazole was isolated, having m. p. 190—191° not depressed by admixture with synthetic *d*-ribobenziminazole; no *d*-arabobenziminazole was obtained.

(iv) Guanylic acid (6·3 g.), prepared according to Buell and Perkins (*J. Biol. Chem.*, 1927, **72**, 21) was heated under reflux for 96 hours with 50% aqueous pyridine (100 c.c.). The solution was then evaporated to dryness under reduced pressure and the residue dissolved in the smallest quantity of hot water. After being thoroughly cooled, the guanosine was crystallised from water, and a sample (3·8 g.) was hydrolysed, the sugar oxidised, and the calcium aldonate condensed with *o*-phenylenediamine exactly as in experiment (iii). *d*-Ribobenziminazole alone was obtained.

(v) Calcium aldonate obtained from guanosine as described in experiment (i) was condensed with *o*-phenylenediamine as in experiment (iii), and yielded only *d*-ribobenziminazole. The same calcium aldonate was then condensed with *o*-phenylenediamine as in experiment (i), but using 1·4 mols. of hydrogen chloride. Both *d*-arabobenziminazole and *d*-ribobenziminazole were isolated and compared with synthetic materials.

Preparation of d-*Ribobenziminazole from Adenosine.*—Adenosine (10 g.), prepared as described by Bredeck, Martini, and Richter (*loc. cit.*) from yeast nucleic acid supplied by The Pharmaco-Chemical Products Co. Ltd., was hydrolysed by the same procedure as that used in experiments with guanosine. The calcium aldonate obtained by oxidising the sugar by the method of Hudson and Isbell (*loc. cit.*) was condensed as already described with *o*-phenylenediamine (1·58 mols.) in presence of hydrogen chloride (2·48 mols.). No arabobenziminazole was isolated. *d*-Ribobenziminazole was obtained having m. p. 190—191°, and did not depress the m. p. of synthetic *d*-ribobenziminazole. In 5% citric acid $[\alpha]_D^{20°}$

was + 21·5°. The picrate and hydrochloride had m. ps. 184—185° and 200—201° respectively, not depressed by admixture with authentic specimens.

Preparation of d-*Ribobenziminazole from Yeast Nucleic Acid.*—(*a*) Yeast nucleic acid (B.D.H.) (40 g.) was refluxed with N/10-sulphuric acid (4 l.) for 6 hours. Colorimetric analysis for inorganic and total phosphate then showed that hydrolysis of the purine nucleotides was complete. Silver sulphate was added to the solution and, after removal of the precipitated silver salts of purines and pyrimidine nucleotides, the filtrate was freed from silver ions by passage of hydrogen sulphide, filtration (charcoal), and aeration, and from sulphate ions by addition of hot saturated baryta and removal of the barium sulphate by filtration. The filtrate, after being concentrated under reduced pressure to 250 c.c., contained 6·2 g. of pentose (Hinton and Macara, *loc. cit.*) and was oxidised as described by Hudson and Isbell, yielding calcium aldonate (6·7 g.). The aldonate (4 g.) was condensed with *o*-phenylenediamine (1·58 mols.) and concentrated hydrochloric acid (2·48 mols. of hydrogen chloride) as previously described. *d*-Ribobenziminazole (1·4 g.) was obtained and had m. p. 190—191° not depressed by admixture with synthetic *d*-ribobenziminazole. The picrate and hydrochloride had m. p. 184—185° and 201—202° respectively, not depressed by admixture with authentic specimens.

(*b*) Repetition of the preceding experiment with yeast nucleic acid supplied by the Pharmaco-Chemical Products Co. Ltd. gave identical results.

(*c*) Yeast nucleic acid (B.D.H.) (50 g.) was refluxed for 4 hours with N-hydrobromic acid (2 l.) and sufficient bromine to saturate the solution. After removal of excess of bromine by distillation, purines, pyrimidine nucleotides, and most of the bromide ions were removed by addition of lead carbonate to the hot solution, which was cooled and filtered from lead bromide. Excess of lead was removed from the filtrate by passage of hydrogen sulphide followed by aeration, and bromide ions were then completely removed by addition of silver carbonate. The filtrate from silver salts was freed from silver with hydrogen sulphide, and, after removal of silver sulphide, the solution was concentrated under reduced pressure to 500 c.c. and refluxed with calcium carbonate for 2 hours. After filtration (charcoal) from excess of calcium carbonate and concentration under reduced pressure to 20 c.c., the solution was poured into 95% ethyl alcohol (500 c.c.). The calcium aldonate was collected and condensed with *o*-phenylenediamine (1·58 mols.) and hydrochloric acid (2·48 mols. of hydrogen chloride) as described above. *d*-Ribobenziminazole was isolated and had m. p. 190—191° not depressed by admixture with synthetic *d*-ribobenziminazole.

Determination of the Loss of Hydrogen Chloride during Condensation.—Calcium *d*-arabonate (4 g.), prepared from *d*(−)-arabinose by oxidation with bromine, was heated at 130—140° for 2 hours with *o*-phenylenediamine (1·48 mols.), concentrated hydrochloric acid (1·3 mols. of hydrogen chloride), water (8 c.c.), and ethyl alcohol (2 c.c.). The syrup was dissolved in water (50 c.c.) and made neutral to litmus by addition of 2N-sodium hydroxide followed by a few drops of aqueous ammonia (*d* 0·880), and after removal of the precipitated arabobenziminazole by filtration the solution was diluted to 60 c.c. and analysed for chloride ions by titration with silver nitrate. The solution contained 0·025 mol. of hydrogen chloride.

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