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The Isolation of Nebularine and the Determination of its Structure

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Nebularine, an antibiotic active against *Mycobacteria* and present in the mushroom *Agaricus (Clitocybe) nebularis* Batsch. has been studied since 1945 [1–4]. In 1952 we isolated nebularine and in a preliminary report [4] we were able to give information on its composition, the structure being formulated as 9-(D-ribosyl)purine. Since then we have demonstrated the compound to be a furanoside. The configuration of the glycosidic centre has not been investigated by us but was demonstrated through the synthesis of nebularine by G. Brown and Weliky [5] at the Sloan-Kettering Institute for Cancer Research in New York. This synthesis shows definitely the correctness of our previous conclusions and in addition that the glycosidic centre has the β-configuration. — We will now describe in detail the isolation of nebularine and the determination of its structure.

The isolation of nebularine from the mushroom may be considered as difficult. The content of nebularine in the fresh mushroom is very low, *i. e.* about 0.002 % *. As we had no chemical test for the identification of the compound, we had to check every fraction against *tubercle bacilli* **. Other difficulties were the high water solubility of nebularine as compared with its low solubility in lipoid solvents, and the impossibility of finding a selective precipitation reaction. — The first steps in the isolation procedure have been described previously [3]. These steps include an aqueous extraction of the mushroom, precipitation of the proteins with acetone, the removal of lipoid matter by extraction with chloroform and with ether, adsorption of nebularine on active carbon followed by elution, and chromatography on aluminum oxide. The fraction thus obtained showed a high biological activity but nevertheless we had the problem of separating nebularine from at least ten amino acids, eight "nucleo" derivatives (purines, pyrimidines, and nucleosides), and a number of other compounds, highly soluble in water. We tried to separate nebularine

* Calcd. not only from the yield but also from the bacteriological activity.

** For the current experiments we used *Mycobacterium avium*, strain Bang, in Dorset medium.

from the amino acids by a simple extraction procedure of the aqueous solution with a suitable organic solvent. But it appeared *a priori* impossible according to the fact that nebularine was extracted together with the amino acids. Finally, however, the problem was solved by shaking out the water solution fifteen times with a solvent mixture chloroform-ethanol 2 : 1 (v/v) *. All the amino acids remained in the water phase whereas all the nebularine had been transferred to the chloroform-ethanol mixture. By using the paper chromatography technique developed by Löfgren [7] for the analysis of purines, pyrimidines, and nucleosides we could show that the chloroform-ethanol extract contained nine nucleo derivatives, *viz.* adenine, adenosine, guanosine, hypoxanthine, cytosine, cytidine, uracil, uridine, and one unknown. In order to find a R_F value of nebularine we ran a one-dimensional paper chromatogram of a special type, the starting line being totally covered with spots. The paper strip was then cut into many parts, each of which was eluted with a 0.2 % solution of 2-octanol in 30 % ethanol. Each fraction was then tested for tuberculostatic activity. Only the region corresponding to the unknown compound gave an active extract. Therefore we were able to conclude with a high degree of probability that our nebularine belonged to the purine or the pyrimidine group.

Thus it seemed possible for us to divide the nucleo derivatives into fractions by means of chromatography on a suitable ion exchanger. The use of Dowex 50 in the hydrogen form seemed to give a good result. The active principle appeared together with adenosine and cytosine on elution with dilute ammonia. On the paper chromatogram, however, the fraction showed a much higher spot intensity than was to be anticipated from the measured tuberculostatic activity. Because of this somewhat confusing result we avoided the ion exchange technique. Later, when we had isolated nebularine and had found purine to be one of its components we could explain the phenomenon. We were then able to demonstrate that nebularine was partly hydrolyzed in contact with the ion exchanger. In the effluent from the column, purine appeared together with nebularine, both of which had the same R_F value in the resolving liquid used.

From the chloroform-ethanol solution the solvents were removed and the residue was distributed between *n*-butanol and water. After separation of the two layers the butanolic solution was extracted three times with water. From the combined four aqueous solutions the water was evaporated and replaced by dry *n*-propanol. Addition of much ether resulted in a solid precipitate which consisted mainly of nucleo derivatives including a relatively small part of the nebularine. The filtrate was found to contain adenine, adenosine, uracil, uridine, most of the nebularine, and some other compounds not belonging to the purine or the pyrimidine group. Redissolving and reprecipitating of the solid fraction gave another filtrate containing some more nebularine. By evaporation of the combined filtrates, a solid crystalline mass was obtained. — In order to get further separation we tried a number of procedures. However, as the usual routine methods failed we applied the countercurrent distribution technique and found it to be successful. The distribution was carried out between *n*-butanol and water. At first we determined the partition coefficient

* Stoll, Renz, and Kreis [6] have used this mixture succesfully in shaking out highly water-soluble glycosides.

Fig. 1. Photomicrograph of crystalline nebularine (8 ×).

of nebularine. For this purpose a distribution was made in a Craig apparatus, the number of transfers being 49. The relative concentrations of nebularine in the tubes were determined by bacteriological experiments and also by estimating spot intensities on paper chromatograms. Both methods gave the same value of the partition coefficient. From the same run, by analysing spot intensities, we also obtained the partition coefficients of adenosine, uracil, and uridine. (As mentioned above, these compounds, and also adenine, were present together with nebularine in the propanol-ether soluble product.) We found the same values as recorded previously by Tinker and Brown [8] who determined the partition coefficients for a number of nucleo derivatives in distributing the compounds between *n*-butanol and a phosphate buffer of pH 6.5. Because of this agreement between their experiments and ours, we took their value for adenine (*cf.* above) without any further experimental checking. With the aid of these values of the partition coefficients we could show by calculation that a distribution carried to 120 transfers should give a fraction containing all the nebularine and uracil (both have the same partition coefficient) and a relatively small amount of the adenosine (for details, see the experimental part). After this distribution had been carried out, the desired fraction was found within the anticipated tube interval. By washing the dry product with ice-cold absolute ethanol most of the impurities could be removed without considerable loss of nebularine. The product was then crystallized from absolute ethanol. The crystals, as shown by paper chromatography, contained no adenosine but a small amount of uracil. A second recrystallization from the solvent mixture ethyl methyl ketone-methanol 11 : 3 (v/v) increased the melting point by 1—2 degrees. Only traces of uracil could be found. Another recrystallization from the same solvent mixture resulted in a product with a sharp melting point and free from uracil. Further recrystallizations did not alter the product as to its melting point and elementary composition.

From the solvent mixture ethyl methyl ketone-methanol (*cf.* above), nebularine is obtained as small rhombohedral crystals (see Fig. 1). The

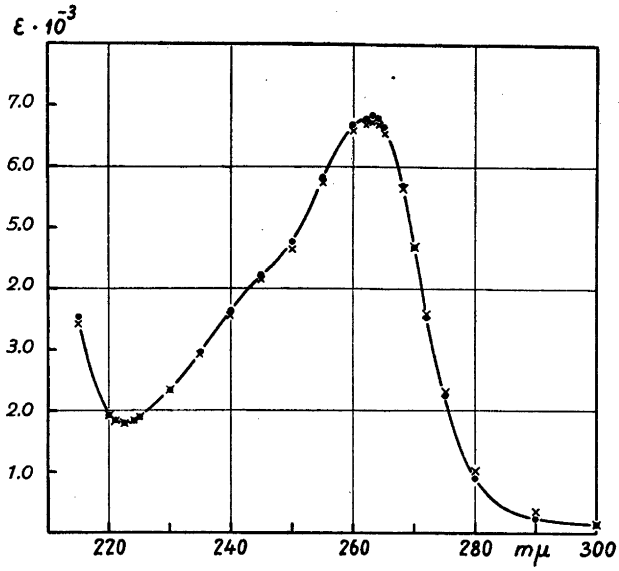


Fig. 2. Ultraviolet absorption of water-solutions of:
—●—●—● *Nebularine from Agaricus (Clitocybe) nebularis Batsch.*
× × × *Synthetic 9-(β-D-ribofuranosyl)purine.*

melting point of the pure nebularine is 181—182° (corr.). Nebularine is considerably soluble in cold water (about 10 %), slightly soluble in cold ethanol, very slightly soluble in acetone and ether, and almost insoluble in chloroform. The specific rotation, $[\alpha]_D^{20}$, in aqueous solution is $-47.5°$ at $c = 2$, and $-47.3°$ at $c = 1$; the difference between the two values might possibly fall within the limits of experimental error. In the ultraviolet, nebularine has a maximum at 263 mμ and a minimum at 222.5 mμ; see Fig. 2, where the absorption curve of the natural product is compared with that of the synthetic compound *. The partition coefficient $C_{n\text{-butanol}} / C_{\text{water}}$ is 0.42. In the solvent mixtures given by Löfgren[7], nebularine has the following R_F values on Whatman No. 4 filter paper at 22° ± 0.5° : 0.73 ("*iso*butyric acid liquid"), 0.52 ("butanol-*iso*butyric acid liquid"), 0.82 ("morpholine liquid"), and 0.66 ("piperidine liquid"). In water-solution, nebularine is markedly thermostable. Thus it endures three hours' heating in aqueous solution at 100° without decomposition (a longer heating time has not been tried). In dilute ammonia solutions the stability may be considered as fairly high (no decomposition in 0.1 *N* ammonia at room temperature after 8 days), but it is less pronounced in aqueous solutions of diethylamine, piperidine, and mineral acids. Nebularine gives a positive Molisch reaction.

The tuberculostatic activity ** on *Mycobacterium avium*, strain Bang, in Dorset medium is 1 : 500 000 but pronounced activity has been observed down

* A sample of the synthetic product was kindly sent to us by Dr. G. Brown.
** The minimal concn. at which growth still can be traced.

to a concentration of 1 : 3 000 000. This effect is noticed during at least 30 days. The effect is about the same for *Mycobacteria* of human and bovine types and for *Mycobacterium phlei*. The compound has no antibiotic effect on the following bacteria: *Sarcina lutea*, *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, and *Bacillus subtilis*. Its bacterial spectrum may therefore be stated as very selective. When tested in tissue culture, nebularine strongly retards the cell growth of both normal mouse embryo skin and sarcoma 180[5]. In considerable dilutions there is some preferential activity against the cancer cells. In experiments with barley seedlings we found nebularine to inhibit the growth almost completely at a dilution of 1 : 3 000. On root tips of *Allium cepa*, nebularine shows inhibition of the cell division, disturbances of the spindle function, and a remarkable chromosome contraction. These effects were demonstrated in the concentration range 0.01—0.001 % *. When tested subcutaneously in white mice, nebularine shows a surprisingly high toxicity, the LD_{50} value being approximately 100 mg / kg. Nebularine has haemolytic activity only at a relatively high concentration (0.5 %).

Elementary analysis of nebularine gave the formula $C_{10}H_{12}N_4O_4$. On hydrolysis two cleavage products were found.

One of them was shown to be identical with purine, the actual compound and synthetic purine having the same melting points, R_F values, and spectra in the ultraviolet. Furthermore the hydrolysis product gave a picrate of the same composition and melting point as purine picrate. The mixed melting point of the two picrates showed no depression. — This is the first time purine has been demonstrated to be a component of a natural product.

The other cleavage product was found to be identical with D(−)ribose. The identity of the two sugars was established by means of paper chromatography, from measurements of optical rotation, and further by comparing the two α,α-benzylphenylhydrazones as to their compositions and melting points, and by determining the mixed melting point.

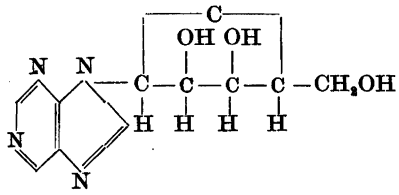
The uptake of one mole of periodate without production of formic acid showed that nebularine has the furanoside structure.

A study of ultraviolet spectra indicated that the ribose is attached at position 9 of the purine nucleus. We planned to make further investigations on this point by comparing the ultraviolet spectra of nebularine, 7-methylpurine and 9-methylpurine, and eventually to synthesize 9-(D-ribofuranosyl)purine, but in the meantime we got the information from Dr. G. Brown at the Sloan-Kettering Institute in New York that he was engaged in synthesizing this nucleoside. We therefore awaited the results from the Sloan-Kettering Institute. Recently Brown and Weliky [5] succeeded with the synthesis of the nucleoside which was shown to have the β-configuration. We sent a sample of nebularine to this institute and by comparing the crystalline forms, melting points, spectra, and biological activity, and by examining the mixed melting point, Brown and Weliky could demonstrate our nebularine to be identical with their synthetic product [5]. The $[\alpha]_D$ value of the synthetic product at $t = 25°$ and at $c = 1$ in aqueous solution was found by Brown and Weliky to be $-48.6°$.

* Experiments performed by B. Kihlman.

Under the same conditions but at $t = 20°$, our measurements on the natural product gave a specific rotation $= -47.3°$.

Thus, it is made clear that nebularine is 9-(β-D-ribofuranosyl)purine:



Nebularine = 9-(β-D-ribofuranosyl)purine

EXPERIMENTAL

*The isolation of nebularine from Agaricus (Clitocybe) nebularis Batsch.**. By means of a mincing machine, 1 130 kg of fresh mushroom were ground and from the slurry thus obtained, the juice was pressed off hydraulically at 150 kg/sq.cm. The resulting firm mass was then suspended in its own weight of water. After some time the liquid was separated as before by expressing. The two solutions were combined and mixed with 2 920 kg of acetone. A protein precipitate formed and the mixture was kept at 0° over-night. Most of the supernatant solution was siphoned off and the remaining mixture filtered with suction. The precipitate was suspended in 75 % acetone and after some time the mixture was filtered at the pump. The filtrate was combined with the former solutions. The acetone and part of the water were distilled off under reduced pressure and at a liquid temperature of 25° (thermometer immersed in the liquid). The distillation was stopped when 778 l of solution remained. This solution which had a dry content of 2.25 % was now extracted five times with 260-l portions of chloroform and three times with 130-l portions of ether. From the aqueous solution the dissolved ether was removed by distillation under reduced pressure. The dry content was adjusted to 2 %. — Under vigorous stirring, this solution was treated with 3.05 kg of active carbon (Merck's "Carbo Activatus" — washed several times with hot water). Stirring was continued for half an hour and the mixture was then kept at 0° over-night. The carbon was sucked off and eluted with 3 × 763 kg of a 0.2 % solution of 2-octanol in 30 % ethanol. During each elution the mixture was stirred mechanically for five hours and then allowed to stand over-night at 0°. The supernatant solution was every time siphoned off from the carbon sediment and then, without further separation, the next portion of eluant was added. After the third and last elution, the separation was completed by centrifugation. The combined eluates were concentrated to a volume of 120 l by distillation under reduced pressure and at a maximum liquid temperature of 30°. — To this solution, 20 kg of aluminium oxide ("Brockmann") ** were added under vigorous stirring which was continued for one hour. The aluminium oxide was allowed to settle for some time and most of the supernatant solution was siphoned off. The remaining mixture was filtered at the pump and the aluminium oxide was washed with 40 l of water. The combined solutions were sucked through a filter covered with pure wet aluminium oxide, the dry weight of which was 3 kg. The oxide layer was then washed with 5 l of water and the filtrates were combined. All these filtrations through aluminium oxide were made in a refrigeration room of approximately 0°. The main part of the water was now distilled off under diminished pressure and at a maximum liquid temperature of 20°. The distillation was stopped when a volume of 40 l remained. — This solution was extracted 15 times with 40-l portions of a solvent mixture chloroform-ethanol 2 : 1 (v/v).

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* Each step in the isolation procedure means an increase in the tuberculostatic activity.

** Previously [3] the purifying step with Al_2O_3 was carried out as a true chromatography. Here the procedure has been simplified.

From a smaller portion of the combined extracts an aqueous solution was now prepared. In analysing this solution for nucleo derivatives by means of paper chromatography and using Löfgren's technique [7], the presence of adenine, adenosine, guanosine, hypoxanthine, cytosine, cytidine, uracil, uridine, and one unknown compound could be demonstrated. Much attention was paid to the latter compound. In the "piperidine liquid" (*cf.* Löfgren [7]) on Whatman No. 4 filter paper at 22°, the unknown compound gave a spot with the R_F value 0.66. Experiments were now made in order to find the R_F value of nebularine. For this purpose sections from paper chromatograms were eluted and the resulting extracts tested for tuberculostatic activity. To each of two filter-paper strips six spots were applied, each containing a relatively high content of substance. The chromatograms were developed by means of the "piperidine liquid". After drying, each of the two papers was cut into six sections, corresponding to the following R_F intervals: 1) 0.00—0.30 2) 0.30—0.57 3) 0.57—0.63 4) 0.63—0.69 5) 0.69—0.75 6) 0.75—1.00. The cuttings with the same numbers were matched and each pair was eluted with a 0.2 % solution of 2-octanol in 30 % ethanol. Only section No. 4 corresponding to the R_F value of the unknown compound gave an active extract when tested for tuberculostatic activity. It was therefore concluded, with a high degree of probability, that the spot with the R_F value 0.66 belonged to nebularine. (The subsequent experiments gave full evidence for this assumption.)

The combined chloroform-ethanol extracts were concentrated by distillation under reduced pressure and at a bath temperature of 40—45° to a volume of 3.6 l. — The residue which consisted of an aqueous solution containing some solid matter was shaken with 3.6 l of *n*-butanol. Most of the solids dissolved in the butanolic phase. The whole mixture was filtered with suction. The butanolic layer, separated from the aqueous solution, was then extracted with its own volume of water and this procedure repeated twice. The combined *four* aqueous solutions were evaporated to dryness under reduced pressure and at a maximum *liquid* temperature of 25°. At the end of the distillation the *bath* temperature was kept at 40°. The residue, finally dried over silica gel in a vacuum desiccator, consisted of 86 g of a light brown mass. — The finely powdered and carefully dried product was divided into four parts. Each portion, weighing 21.5 g, was mixed with 700 ml of dry *n*-propanol in a round-bottomed flask equipped with a condenser and a separatory funnel, both fitted with a calcium chloride tube, and a mercury-sealed Hershberg stirrer. The powder was dissolved by heating the flask in a paraffin oil bath which was maintained for some time at 70°. When the material had gone into solution, heating was discontinued without removing the oil bath. As soon as the temperature of the oil bath had fallen to 35—40°, 3.5 l of absolute ether were introduced under vigorous stirring and during a period of four hours. A yellow precipitate formed. The flask was carefully closed and kept at 0° over-night. The precipitate was sucked off and the filtrate evaporated to dryness under reduced pressure and at a maximum bath temperature of 40°. The residue consisted of a yellowish syrup which after further drying over silica gel in a vacuum desiccator soon solidified to a crystalline mass. The amount deposited from the four runs was 57.2 g. From the combined four precipitates an additional 7.8 g was obtained by redissolving in propanol and reprecipitating with ether — the yield from the whole procedure thus being 65 g. By analysis of this highly active product for nucleo derivatives it was found to contain nebularine contaminated with adenine, adenosine, uracil, and uridine.

The propanol-ether soluble product was then purified by means of countercurrent distribution between *n*-butanol and water. At first the partition coefficient was determined by distributing a small amount of the product in a Craig apparatus, made up of small compartments. The number of transfers was 49. When the distribution was completed all fifty water-phases were tested for tuberculostatic activity. The maximum activity was found in the tubes 13—16. The position of maximum concentration was also investigated by analysis of spot intensities on paper chromatograms (visual estimation of fluorescence intensity). The same result was obtained. The tube number of maximum concentration was therefore set to 14.5 corresponding to a partition coefficient $C_{\text{butanol}}/C_{\text{water}} = 0.42$. Estimation of spot intensities also gave the partition coefficients of adenosine, uracil, and uridine. The corresponding value for adenine was taken from Tinker and Brown [8] who distributed a number of nucleo derivatives between *n*-butanol and a phosphate buffer of pH 6.5 (*cf.* above). Thus all necessary figures were ascertained in order to calculate the possibility of separating nebularine from the other nucleo derivat-

ives having the following partition coefficients: adenine 2.77, adenosine 0.76, uracil 0.40, uridine 0.12. Calculations on a countercurrent distribution * carried to 120 transfers showed that nebularine should occur in the tubes 22—48. In this calculation the loss in yield was set to 1 %. Furthermore it was calculated that in this interval no adenine would interfere, whereas a little of the uridine, some of the adenosine and all the uracil would contaminate nebularine. (Nebularine and uracil have approximately the same partition coefficients; *cf.* above.) However, it could also be anticipated that a purer product might be obtained from the tubes 27—44, since in this case all uridine and more of the adenosine should be avoided. In other words: in carrying out 120 transfers, the selection of tubes 27—44 might result in a fraction containing almost all of the nebularine and uracil, little of the adenosine (about 8 % of its starting amount), and none of the other compounds. This scheme was followed in the procedure. A big Craig apparatus ** in which each compartment had a capacity of 2 × 250 ml was used. Of the available starting material (65 g) one third was used in each of three distribution procedures. In agreement with the calculations the maximum concentration of nebularine occurred in the compartments 35—36. The contents of compartments 27—44 were mixed together and evaporated to dryness under reduced pressure and at a maximum *liquid* temperature of 25°. At the end of the distillation the *bath* temperature was kept at 40°. The residue consisted of a pale yellow syrup which after further drying over sulphuric acid in a vacuum desiccator soon solidified to a crystalline mass. From the 65 g of starting material, 29.9 g were obtained. This product was analyzed for nucleo derivatives by means of paper chromatography and proved to contain the expected compounds, *i. e.* nebularine, uracil, and adenosine. As to the proportions, nebularine predominated over the two others — the content of adenosine being small.

To the finely divided and carefully dried product (29.9 g), 120 ml of ice-cold absolute ethanol were added and the mixture was kept at 0° for 18 hours. The powder was sucked off and once more treated with ethanol in the same way. The remaining, almost white crystalline powder weighed 23.4 g. An analysis of this product as well as of the combined light yellow alcoholic solutions showed that almost all of the nebularine had been retained in the powder, whereas the main part of the uracil together with some of the adenosine had gone into solution. The powder thus contained nebularine contaminated by small amounts of uracil and adenosine. The product was now recrystallized from absolute ethanol. From this operation 17.6 g of small prisms melting at 178—180° (corr.) were obtained. In this product no adenosine could be detected but a small amount of uracil. For further purification, 3.00 g were recrystallized from a solvent mixture ethyl methyl ketone-methanol 11 : 3 (v/v). The yield was 2.13 g of a product melting 1—2 degrees higher. Only traces of uracil could be found. Another recrystallization from the same solvent mixture resulted in a product melting at 181—182° (corr.) and free from uracil. Further recrystallizations did not alter the product as to its melting point and elementary composition. From the solvent mixture ethyl methyl ketone-methanol the pure nebularine appeared in the form of small rhombohedral crystals (Fig. 1). (Found: C 47.7, H 4.83, N 22.0, O 25.4 ***.) Calc. for $C_{10}H_{12}N_4O_4$ (252.2): C 47.6, H 4.80, N 22.2, O 25.4.)

Optical rotation of nebularine. A solution of $c = 2$ was prepared by dissolving 400.0 mg of nebularine in water to a total volume of 20.00 ml. The rotation for the D line at $l = 2.00$ dm and $t = 20.0°$ was found to be $-1.90°$. After dilution of this solution to $c = 1$ the rotation was again determined and found to be $-0.945°$. Thus for $c = 2$, $[\alpha]_D^{20} = -47.5°$ and for $c = 1$, $[\alpha]_D^{20} = -47.3°$.

Spectrum of nebularine in the ultraviolet. The measurements were made in aqueous solution by using a Beckman quartz spectrophotometer. Fig. 2 shows the absorption curves of natural nebularine and of the synthetic product (the latter sent to us by Dr. G. Brown). The coinciding curves have a maximum at 263 mμ and a minimum at 222.5 mμ.

Purine obtained from nebularine by hydrolysis. Nebularine, 19.0 mg, was dissolved in one ml of 3 % hydrochloric and the mixture kept at 80° for one hour in a water bath. The solution was then neutralized with ammonia and poured on a column of Dowex 50

* Concerning such calculations, see for instance Nichols [9].

** The type of the glass vessels was similar to that given by Craig, Gregory, and Barry [10].

*** By means of the Unterzaucher method.

in the hydrogen form (particle size, 70—100 mesh U.S. series; diameter and height of the column, 7 and 40 mm respectively). Frequent microchemical tests for sugars (Molisch) and for nucleo derivatives (fluorescence of spots on filter paper) were carried out on the effluent. Very soon a sugar fraction appeared. The column was washed with somewhat more water than was necessary to obtain a negative Molisch reaction. Then the column was eluted with 0.2 *N* ammonia. After some time and within a short period, a fraction appeared which was positive in the spot test on filter paper (*cf.* above). The eluate was evaporated to dryness. The brownish crystalline residue contained no nebularine and melted at about 204° (corr.). Yield 8.7 mg (97 % assuming the product to be purine formed from ribosyl purine). The product was now extracted with 0.5 ml of boiling toluene. The resulting solution was decanted from the solid material and yielded crystals on cooling. The extraction procedure was repeated five times, each time using the mother liquor. The insoluble residue consisted of a black deposit. The total yield of fine colourless needles melting at 216—217° (corr.) was 4.8 mg. The melting point is in agreement with that of purine given by Fischer [11].

The substance gave the same R_F values as synthetic purine. Thus, in the solvent mixtures given by Löfgren [7], both purine and the hydrolysis product were found to have the following R_F values on Whatman No. 4 filter paper at 22.0° ± 0.5° : 0.82 ("*iso*butyric acid liquid"), 0.68 ("butanol-*iso*butyric acid liquid"), 0.82 ("morpholine liquid"), and 0.66 ("piperidine liquid").

Another evidence for the identity of the cleavage product with purine was established in comparing their maxima and minima in different media in the ultraviolet. Maxima and minima were measured in neutral, acidic, and alkaline solutions, using a Beckman instrument. Synthetic purine and the hydrolysis product gave the same values as shown in Table 1. (No spectral data of purine are available from the literature.)

Table 1. Maxima and minima in the ultraviolet of synthetic purine and of the cleavage product from nebularine. Measurements in neutral, acidic, and alkaline media; λ in mμ.

Medium	λ_{max}	λ_{min}
Water	262.5	221
1 *N* HCl	260	229
1 *N* NaOH	271	236

For further characterization the picrate was prepared: In one drop of water, 3 mg of substance were dissolved and then 0.5 ml of a saturated aqueous solution of picric acid was added. The separated needles were recrystallized from water. The melting point was found to be 205—208° (corr.), in agreement with the recorded melting point of purine picrate [11]. When mixing synthetic purine picrate with the actual picrate, no depression of the melting point occurred. (Found: C 37.8, H 1.84, N 28.3. Calc. for $C_{11}H_7N_7O_7$ (349.2): C 37.8, H 2.02, N 28.1.)

D(—)*Ribose obtained from nebularine by hydrolysis.* In 5 ml of 0.1 *N* sulphuric acid, 49.9 mg of nebularine were dissolved and the solution was kept at 100° for four hours. After cooling, the solution was neutralized to pH 7 with dilute ammonia and then poured on a column of Dowex 50 in the hydrogen form (particle size, 70—100 mesh U.S. series; diameter and height of the column, 7 and 40 mm respectively). Frequent microchemical tests for sugars (Molisch reaction) and for nucleo derivatives (*cf.* above) were carried out on the effluent. Purine and unchanged nebularine were adsorbed on the resin, whereas a sugar fraction passed through. This fraction was taken up in a mixture of water and freshly prepared barium carbonate. The column was washed with somewhat more water than was necessary to obtain a negative Molisch reaction. The solid material ($BaSO_4$ + $BaCO_3$) was then filtered off with suction and washed with water. The combined filtrate and washings were evaporated to dryness under reduced pressure. A syrup containing some barium carbonate was obtained. This mixture was extracted with a small amount of hot absolute ethanol. The barium carbonate was filtered from the solution and washed with a little hot alcohol. From the combined filtrate and washings the alcohol was evaporated over silica gel in a vacuum desiccator. The residue consisted of a crystalline mass. Yield 22.6 mg (76 % assuming the product to be ribose formed from ribosyl purine).

When Bial's test was carried out, the product gave a positive reaction on pentoses and of exactly the same type as found in a parallell test made with commercial ribose. Spot tests on filter paper using Partridge's naphtoresorcinol reagent [12] gave a negative reaction for ketoses.

For further characterization of the sugar, paper chromatography was applied. A technique developed by Löfgren * was used.

The irrigating liquid used in this technique is made up of 60 ml of ethyl acetate, 17 ml of glacial acetic acid, and 17.5 ml of water. This mixture has a high resolving power on sugars. In order to enhance the resolution the mobile phase is allowed to run off the lower edge of the strip (Whatman's No. 4 filter paper; length 57 cm). Thus the chromatogram is run over a rather long period (usually 21 h 45 min.). Under these conditions it is, for instance, possible to separate a mixture of the four aldopentoses into individual components. Rhamnose which travels rapidly on the chromatogram has been used as a standard. Thus a sugar is characterized by its R_r value, *i. e.* the ratio between the distance the sugar travels and the distance through which the rhamnose has moved. The R_r values for the aldopentoses at a temperature of 20.0° ± 0.3° are the following: arabinose 0.62, xylose 0.70, lyxose 0.79, and ribose 0.87.

Using chromatograms of this type, the sugar originating from nebularine was compared with the four aldopentoses. After developement of the chromatograms the spots were revealed by means of Horrocks' benzidine reagent [13]. Each of the four aldopentoses occurred at different positions (*cf.* the R_r values given above). The hydrolysis product from nebularine gave only one spot and at the same position as ribose.

Measurement of the optical rotation in aqueous solution gave $[\alpha]_D^{20} = -19.4° \pm 1.5°$ ($c = 2.8$). For D(−)ribose in water $[\alpha]_D^t$ values corresponding to somewhat higher temperatures than 20° are available from Kuhn *etal.*[14] By extrapolation from these values **: $[\alpha]_D^{20} = -19.9° \pm 0.1°$; *cf.* the recorded value [16] for L(+)ribose: $[\alpha]_D^{20} = +20.7°$ ($c = 4$).

Furthermore, the *α,α*-benzylphenylhydrazone was prepared. In a test tube 16.2 mg of the hydrolysis sugar were dissolved in one ml of ethanol and then a solution of 80 mg of *α,α*-benzylphenylhydrazine in one ml of ethanol was added. The mixture was kept in a boiling water bath for 35 minutes. During this time almost all of the ethanol evaporated. On cooling the syrup crystallized. The crystals were washed carefully three times with 2.5−ml portions of a mixture ether-petroleum ether 1 : 1 (v/v). The yield was 32.6 mg (91 %, when the calculation is based on ribose) of a product melting at 121−124°. Recrystallization from *iso*butanol gave a product (21 mg) with a melting point 128−129° (corr.). This melting point is approximately the same as recorded for the corresponding hydrazones of D(−)ribose *** and D(−)lyxose ****, both of which were prepared. When admixing D(−)ribose benzylphenylhydrazone with the actual hydrazone, no depression in the melting point was found. Admixture with the lyxose derivative gave a large depression of about 30°, when equal parts were mixed. (Found: C 65.0, H 6.74. Calc. for $C_{18}H_{22}N_2O_4$ (330.4): C 65.4, H 6.71.)

Periodate oxidation of nebularine *****. In a 250−ml volumetric flask 100.9 mg of nebularine (0.4000 millimole) were dissolved in 150 ml of water. Then 5 ml of 0.2500 *M* $NaIO_4$ were added and the solution diluted to the mark. After 24 and 45 hours, 50−ml aliquots were taken out with a pipette and the unchanged metaperiodate was estimated iodometrically by the method of Barneby [20] using 0.1000 *N* sodium arsenite. In both cases 3.28 ml of arsenite solution, corresponding to a consumption of 1.07 moles of periodate per mole of nebularine, were needed. The remainder in the flask was investigated as to its content of formic acid (*cf.* Jackson and Hudson [21]). No formic acid could be found.

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* A thorough description of this method and its possibilities of separating sugars will be published later.

** From Kuhn *et al.*[14]: $[\alpha]_D^{24} = -25°$ ($c = 0.9$), $[\alpha]_D^{23} = -23.7°$, $[\alpha]_D^{22} = -22.5°$ ($c = 0.9$). By extrapolation from these values: $[\alpha]_D^{20} = -19.9° \pm 0.1°$. Levene and Jacobs [15] give a value $[\alpha]_D = -19.5°$ but they do not state any temperature.

*** 129° (uncorr.) according to Haiser and Wenzel [17].

**** 128° (corr.) according to Ruff and Ollendorff [18].

***** The general method given by Lythgoe and Todd [19] was followed.

SUMMARY

Nebularine, *i.e.* 9-(β-D-ribofuranosyl)purine, is a naturally occurring compound with strong inhibitory action on cell growth and on *Mycobacteria*. The isolation of nebularine from a mushroom, *Agaricus (Clitocybe) nebularis* Batsch., and the determination of its structure have been described.

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