[CONTRIBUTION FROM THE MERCK SHARP & DOHME RESEARCH LABORATORIES, DIVISION OF MERCK & CO., INC., RAHWAY, NEW JERSEY]

Studies on the Antiviral Agent Helenine. Purification and Evidence for Ribonucleoprotein Nature

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A fractionation procedure is described for purification of the antiviral substance, helenine. The method involves extraction of the mycelium of *Penicillium funiculosum* with neutral buffer containing Mg^{++} and glycerol, precipitation with acetone, ultracentrifugation to sediment the helenine and finally chromatography on ECTEOLA-cellulose. The final product has the properties of a nucleoprotein.

Helenine, a product of the mold *Penicillium funiculosum*, has been reported by Shope[1] to be therapeutically effective in mice against Columbia SK encephalomyelitis and Semliki Forest viruses. Cochran and Francis[2] have also shown that the agent successfully prevents development of poliomyelitis in monkeys. We wish to describe our experiences with the purification of helenine and discuss the evidence which suggests that it is nucleoprotein in nature. A preliminary report of this work has already appeared.[3]

Experimental and Results

Assay.—Fractionation was followed by means of a mouse assay. Helenine samples were administered to mice infected with a lethal dose of SK encephalomyelitis virus and the increase in survival time of treated animals over that of untreated ones was used as an indication of potency. The numerical value, calculated from the survival data, is referred to as the survival index. An index of from 1.0 to 1.6 was considered inactive, and of 1.7 and above, active. The assay was essentially that described by Shope,[1a] but the method has been modified by McClelland[4] to obtain more definitive assay values. A 24-hour pre-treatment dose was substituted for the 24-hour post treatment dose, the observation period was extended to 15 days and survivors were evaluated statistically. The amount of material administered was based on dry weight determinations of samples dialyzed free of salts.

Since various polysaccharide and lipopolysaccharide preparations have been shown to elicit a host-resistance response in animals,[5,6] a few preparations of this nature were tested in the helenine assay. The preparations were (1) zymozan, (2) capsular polysaccharide from Friedländer bacillus type B, (3) lipopolysaccharide from *Escherichia coli* (Strain 08,)[7] (4) lipopolysaccharide derived from *Serratia marcescens* and (5) material prepared from *P. funiculosum* by the Westphal[8] procedure for isolation of lipopolysaccharide. All five preparations were inactive at a dosage of 25–100 μg.

Fermentation.—The mold *Penicillium funiculosum* was used as the source of helenine. The culture medium described by Shope[1a] for growing the organism produced highly active material, but the batch variation was very great with large-scale fermentations. McDaniel and Woodruff of these Laboratories have found that greater consistency could be obtained by use of a less highly purified fermentation medium.[9] Submerged fermentations, which permit the growing of large quantities of the mold, could be carried out with this medium. A 5-liter fermentation yielded about 125 g. of mycelium (75% moisture).

Extraction.—Significant amounts of helenine were found only in the mycelium, indicating that the agent was not actively excreted into the fermentation medium. The mycelium was harvested by filtration on a suction funnel, and the spent broth was discarded. In the early stages of our studies, the extractions were made using 0.04 *M* phosphate buffer *p*H 8. However, when a nucleoprotein structure for helenine was suggested, the extraction was changed to a type used for the isolation of such material. Phosphate or tris-(hydroxymethyl)-aminomethane buffer, each 0.005 *M*, *p*H 7, and containing 0.005 *M* $Mg(C_2H_3O_2)_2$, were two of the buffers that greatly improved the yield of active material. More recently we found that addition of 10% (w./v.) glycerol to the buffer had a definite stabilizing effect on helenine. The dissociation noted during ultracentrifugation was largely overcome when glycerol was present. The phosphate–Mg^{++}–glycerol buffer is now used routinely throughout the fractionation procedure, and purified preparations are stored in this buffer mixture. Penicillin and streptomycin were added to control bacterial growth.

For the extraction, approximately 150 g. (moist weight) of mycelium, as obtained from the filter, was homogenized for 5 minutes in a Waring blendor with 1 liter of the phosphate–Mg^{++}–glycerol buffer mixture. The extract was then separated from the mycelium by centrifugation. All these processes were done at 5°. This extract was quite stable if stored in the cold (5°).

Acetone Precipitation.—Shope[1a] observed that helenine could be precipitated from cell extracts by addition of an equal volume of acetone. We too have found this to be a convenient method of concentrating the activity after extraction. To minimize the denaturation of helenine, the precipitation was done at 0–2°. The precipitated material was collected by centrifugation and suspended in a minimum of the glycerol buffer mixture. Insoluble material was invariably present and was removed by centrifugation. The term acetone precipitate as used below will actually refer to that part of the precipitate soluble in buffer. About 500 mg. of this material was obtained from a 5-liter fermentation.

When tested in the mouse assay, the acetone precipitate usually had a survival index of about 2.0 at a 2.5-mg. dose. A significant number of survivors was often obtained even with this crude material. As demonstrated by Shope,[1c] the active ingredient of the preparation was non-dialyzable.

Preparative Ultracentrifugation.—During ultracentrifugation experiments on the acetone precipitate, it was found that the active constituent could be readily sedimented. Centrifugation was carried out at 5–10° in a Spinco model E ultracentrifuge using either the A or K rotor. A 2-hr. centrifugation at 100,000 × g was sufficient to sediment helenine. The supernatant fluid was then decanted from the sedimented pellet and discarded. No significant amount of activity was ever found in this crude supernatant liquid. The pellet was suspended in the glycerol buffer mixture, about 1/10 the volume of the original solution being used, and the sample was again ultracentrifuged as above. The procedure was repeated a third time and the resulting pellet

(1) (a) R. E. Shope, *J. Exp. Med.*, **97**, 601 (1953); (b) *ibid.*, **97**, 627 (1953); (c) *ibid.*, **97**, 639 (1953).

(2) K. W. Cochran and T. Francis, Jr., *J. Pharmacol. Exp. Therap.*, **116**, 13 (1956).

(3) U. J. Lewis, E. L. Rickes, L. McClelland and N. G. Brink, THIS JOURNAL, **81**, 4115 (1959).

(4) The details of the assay procedure will be published elsewhere by one of us (L. McC.).

(5) D. Rowley, *Brit. J. Exp. Path.*, **37**, 223 (1956).

(6) R. R. Wagner, R. M. Snyder, E. W. Hook and C. N. Luttrell, *J. Immunology*, **83**, 87 (1959).

(7) We wish to thank Dr. D. E. Wolf of these Laboratories for supplying us with this sample of lipopolysaccharide.

(8) O. Westphal, O. Lüderitz and F. Bister, *Z. Naturforsch.*, **7B**, 148 (1952).

(9) The most satisfactory medium found had the following composition (g./l.): glycerol 20, soya peptone 5, Difco peptone 5, yeast extract 3, meat extract 3. We wish to thank Drs. L. E. McDaniel and H. B. Woodruff for this microbiological study.

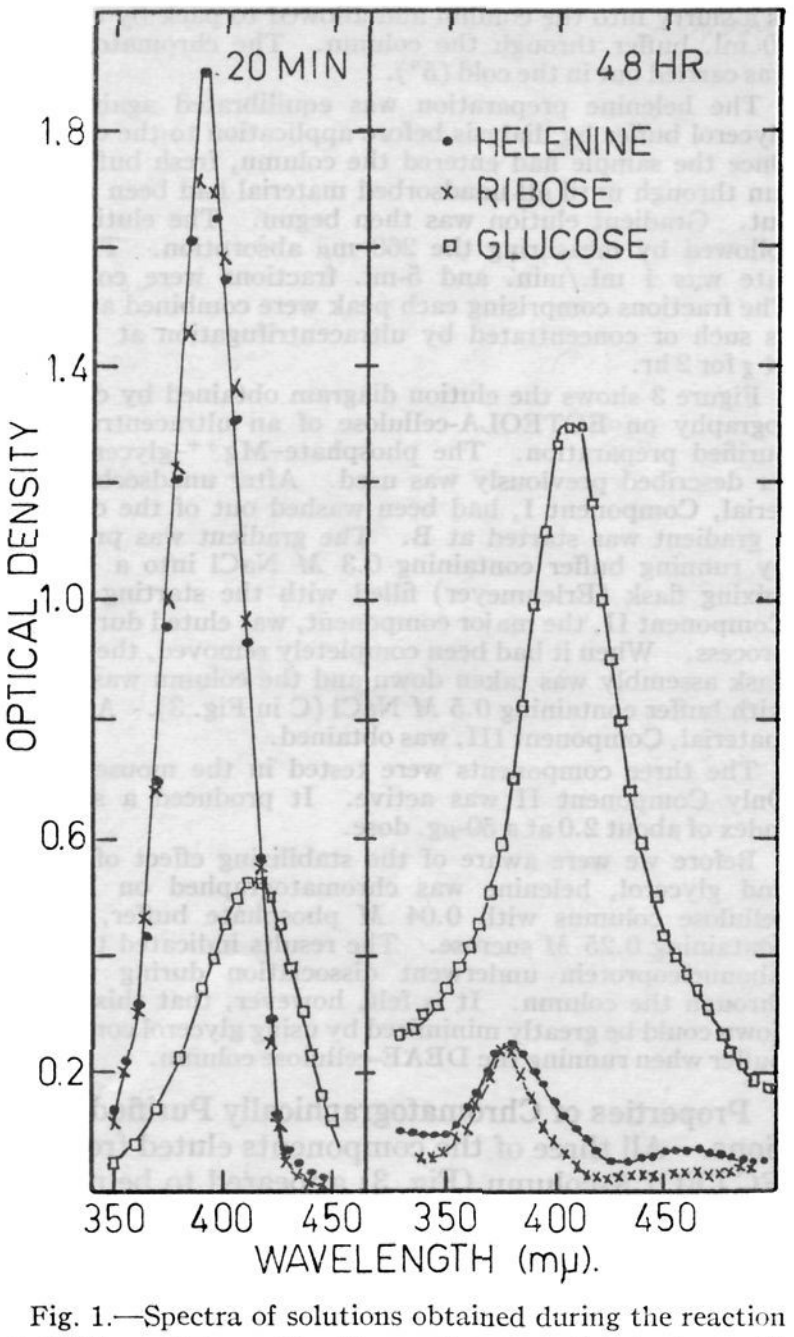

Fig. 1.—Spectra of solutions obtained during the reaction of H_2SO_4–cysteine with ribose, glucose and helenine. 80 μg. of ribose, 87 μg. of glucose and 720 μg. of helenine were used. The left-hand portion shows the curves that were obtained 20 min. after the addition of cysteine; the curves on the right are for the same solutions after standing 48 hr.

taken up in the glycerol buffer mixture. About 25 mg. of pellet was obtained from 1 gram of acetone precipitate. The sedimented material was usually hazy when suspended, but the solution could be clarified by moderate centrifugation (7,000 × *g* for 10 min.). Material prepared in this way had a survival index near 2.0 at a 50- to 100-μg. dose.

Properties of Material Purified by Ultracentrifugation.—A well-defined 260-mμ absorption peak was noted for this fraction. It contained protein[10] and gave a pentose test with orcinol[11] and with H_2SO_4–cysteine.[11] A negative deoxypentose test was obtained.

No hexose contamination was detected during the orcinol test, but approximately 1% non-pentose carbohydrate was estimated to be present from the results of the H_2SO_4–cysteine reaction. As illustrated in Fig. 1, ribose is known to give a sharp absorption peak with a maximum at 390 mμ after a 20 min. reaction with H_2SO_4 and cysteine, while glucose shows a broader peak with a maximum at 410 mμ. When tested under the same conditions, helenine gave an absorption curve indistinguishable from ribose. No skewing in the 410-mμ region was seen. After the solution stood for 48 hr., the intensity of the glucose absorption

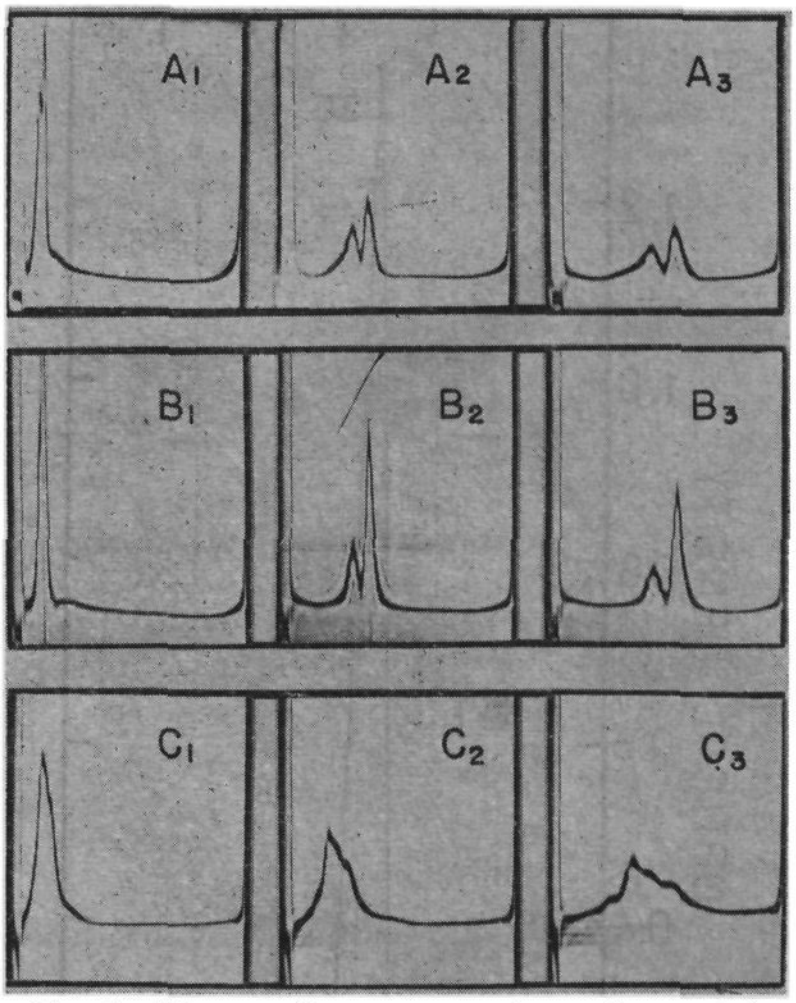

Fig. 2.—Ultracentrifuge patterns for various helenine preparations. A, helenine purified by acetone precipitation and ultracentrifugation; B, helenine further purified by chromatography on ECTEOLA-cellulose (column component II). A and B runs were carried out in 0.004 *M* phosphate buffer, *p*H 7, containing 0.005 *M* $Mg(C_2H_3O_2)_2$ and 10% glycerol. C, material prepared as in A but in the absence of glycerol. The analysis was performed in the same buffer as A and B but no glycerol was present. In each case the photographs were taken 8, 32 and 48 min. after attaining a rotor speed of 25,980 r.p.m. The concentration of the samples were 0.3, 0.5 and 0.6%, respectively, for A, B and C.

increased and the ribose and helenine absorption decreased. It is important to note in this case that ribose gave a symmetrical curve while a definite skewing in the 410–mμ region was seen in the helenine curve. Assuming that the skewing was caused by hexose contamination, it can be estimated that the equivalent of 1% glucose was present in the helenine sample. This is the lowest hexose value obtained with a number of different preparations of comparable biological activity. Material purified by the same procedure has given higher 410–mμ absorption in a number of cases. Thorough dialysis against water before carrying out the H_2SO_4–cysteine reaction seems to eliminate much of the 410-mμ absorption.

For the detection[12] of purines and pyrimidines, a dilute solution of helenine dialyzed free of salts was dried by heating in a boiling water bath under a steam of nitrogen. The dry weight of sample was 3.9 mg. To this was added 0.04 ml. of 70% $HClO_4$ and after mixing, the sample was heated in a glass-stopped tube for one hour at 100°. Then 0.2 ml. of water was added with mixing and the tube was centrifuged. The clear amber supernatant was used for analysis by paper chromatography.

The $HClO_4$ hydrolysate was chromatographed[12] on paper with 2-propanol–HCl–H_2O. From the position on the chromatogram, as well as by comparison of the spectra of the eluted spots with knowns, the presence of guanine, adenine, cytosine and uracil was indicated.

(10) E. W. Sutherland, C. F. Cori, R. Haynes and N. S. Olsen, *J. Biol. Chem.*, **180**, 825 (1949).

(11) Z. Dische in "The Nucleic Acids," edited by E. Chargaff and J. N. Davidson, Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 285.

(12) A. Bendich in "Methods in Enzymology," edited by S. P. Colowick and N. O. Kaplan, Vol. III, Academic Press, Inc., New York, N. Y., 1957, p. 715.

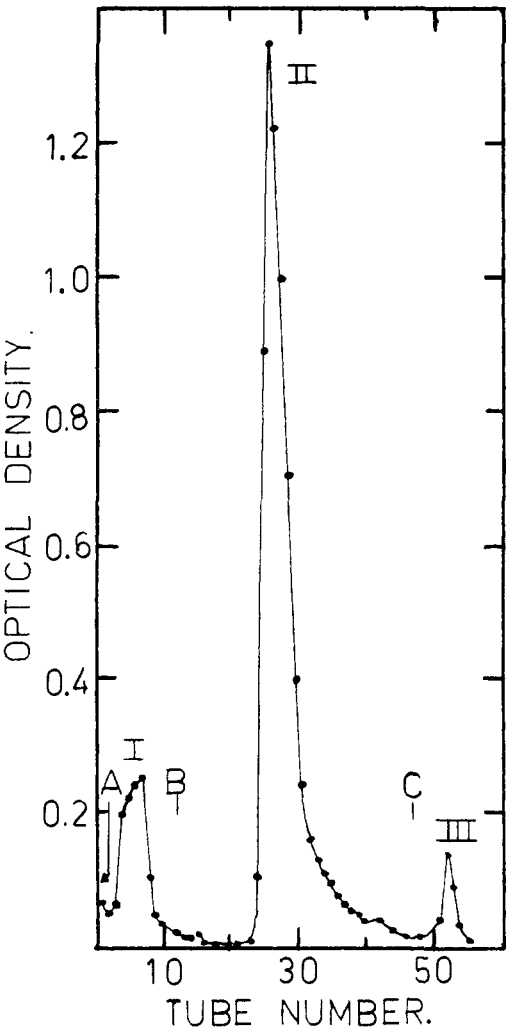

Fig. 3.—Elution diagram obtained with an ECTEOLA-cellulose column. Buffer used was 0.004 *M* phosphate, *p*H 7, containing 0.005 *M* $Mg(C_2H_3O_2)_2$ and 10% glycerol. Elution schedule: A, phosphate–Mg^{++}–glycerol buffer; B, NaCl gradient begun (0.3 *M* NaCl limit); C, column eluted with 0.5 *M* NaCl. See text for explanation of numerals I, II and III.

In the analytical ultracentrifuge, two major peaks and one minor one (Fig. 2A) were observed. The patterns shown were obtained with a rotor speed of 25,980 r.p.m. (Model E, Spinco ultracentrifuge). When the slowest sedimenting boundary had reached the bottom of the cell, the rotor speed was increased to 59,780 r.p.m. No additional components were observed. The sedimentation constants ($S_{20,w}$) of the three peaks (Fig. 2A) were 64, 81 and 133 *S*. We have noted that the sedimentation constant has a significant concentration dependence. Therefore, the *S* values reported are valid only for the concentration indicated in the legend for Fig. 2.

Chromatography.—Since the helenine prepared by ultracentrifugation was heterogeneous during electrophoresis, further purification on columns of modified cellulose was tried. ECTEOLA-cellulose[13] and diethylaminoethyl (DEAE)-cellulose,[13] prepared by the method of Ellis and Simpson,[14] were the two exchangers employed.

Usually about 50 mg. of sample was chromatographed on a 1 × 4-cm. column. The exchanger was equilibrated by repeated washing with the glycerol–buffer and then poured as a slurry into the column and allowed to pack by running 50 ml. buffer through the column. The chromatography was carried out in the cold (5°).

The helenine preparation was equilibrated against the glycerol buffer by dialysis before application to the column. Once the sample had entered the column, fresh buffer was run through until all unadsorbed material had been washed out. Gradient elution was then begun. The elution was followed by measuring the 260-mμ absorption. The flow rate was 1 ml./min. and 5-ml. fractions were collected. The fractions comprising each peak were combined and held as such or concentrated by ultracentrifugation at 100,000 × *g* for 2 hr.

Figure 3 shows the elution diagram obtained by chromatography on ECTEOLA-cellulose of an ultracentrifugally purified preparation. The phosphate–Mg^{++}–glycerol buffer described previously was used. After unadsorbed material, Component I, had been washed out of the column, a gradient was started at B. The gradient was produced by running buffer containing 0.3 *M* NaCl into a 125-ml. mixing flask (Erlenmeyer) filled with the starting buffer. Component II, the major component, was eluted during this process. When it had been completely removed, the mixing flask assembly was taken down and the column was eluted with buffer containing 0.5 *M* NaCl (C in Fig. 3). A cloudy material, Component III, was obtained.

The three components were tested in the mouse assay. Only Component II was active. It produced a survival index of about 2.0 at a 50-μg. dose.

Before we were aware of the stabilizing effect of Mg^{++} and glycerol, helenine was chromatographed on DEAE-cellulose columns with 0.04 *M* phosphate buffer, *p*H 7, containing 0.25 *M* sucrose. The results indicated that the ribonucleoprotein underwent dissociation during passage through the column. It is felt, however, that this breakdown could be greatly minimized by using glycerol containing buffer when running the DEAE-cellulose column.

Properties of Chromatographically Purified Fractions.—All three of the components eluted from the ECTEOLA-column (Fig. 3) appeared to be nucleoproteins. The ultraviolet absorption spectra of the three were not identical. Only component II showed a definite maximum at 260 mμ while component I showed a plateau in the 260–280 mμ region. Component III was too cloudy to give a meaningful spectrum. The 260 mμ/280 mμ ratios for components I and II were 1.23 and 1.36, respectively, in this case. However, a ratio as high as 1.8 has been noted for other active preparations.

Components I and II (Fig. 3) gave positive pentose tests with orcinol and the H_2SO_4–cysteine reaction. Protein was present in both components. Consistent protein values have not been obtained with a number of similarly prepared fractions. At present, it can be stated only that the active helenine preparations contain between 40 and 60% protein. The diphenylamine test for deoxyribose was negative for components I and II.

A $HClO_4$-hydrolysate of chromatographic component II prepared as described previously for material purified by ultracentrifugation showed the presence of guanine, adenine, cytosine and uracil when chromatographed on paper using the 2-propanol–HCl–H_2O system.

Because of the quantity of material available, only component II was analyzed in the ultracentrifuge. The pattern obtained (Fig. 2B) was almost identical to that observed for the starting material applied to the column (Fig. 2A). The only difference was a change in the relative concentrations of the two major peaks. This may be a result of a monomer-dimer relationship which has

(13) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(14) S. Ellis and M. E. Simpson, *J. Biol. Chem.*, **220**, 939 (1956).

already been suggested[15] for a bacterial ribonucleoprotein. The $S_{20,w}$ values for the three components were 71, 87 and 345S.

Stability.—Highly purified helenine preparations lost their biological activity over a period of two or three weeks when stored in 0.04 *M* phosphate buffer, *p*H 7. A clarified solution gradually became cloudy even at 5°. The presence of Mg^{++} and 0.25 *M* sucrose delayed this reaction, but still over a period of a month the solution became turbid and the potency dropped. Storage in 10% glycerol greatly minimized this deterioration, and the biological activity was maintained for at least 2 months. The stabilizing effect of glycerol on the nucleoprotein is clearly demonstrated in the ultracentrifuge patterns shown in Fig. 2C. As mentioned previously, 2A and 2B represent samples that were held in contact with glycerol. Figure 2C is a pattern of a sample processed and analyzed in the ultracentrifuge with the phosphate-Mg^{++} buffer without glycerol present. At least six components can be seen, with $S_{20,w}$ values of 13, 31, 43, 52, 62 and 100 *S*, indicating a disintegration of the nucleoprotein. Material of this nature has activity comparable to the 3-component samples. Since the assay cannot significantly indicate a potency change which is less than 2-fold, no definite statement can be made concerning the minimum fragment size that is needed for activity.

It was interesting to observe how the sample that showed six components during ultracentrifugation (Fig. 2C) behaved upon electrophoresis. A Spinco moving boundary apparatus (Model H) was used. As seen in Fig. 4, electrophoresis showed the presence of one major peak, comprising 90% of the sample and two small components, each about 5%.

Treatment of helenine with ribonuclease did not alter the activity significantly. However, ribonucleoproteins have been shown[16] to be considerably more stable than free nucleic acid toward the enzyme.

As reported by Shope,[1c] helenine is inactivated by heating and lyophilization. We have also observed this. The process of freezing and thawing also brings about a loss of activity, being more pronounced with highly purified material. Crude extracts and the acetone-precipitated material were much less prone to inactivation.

Other Fractionation Procedures.—Of the many fractionation methods studied in addition to those described above, a few were successful and seem worth mentioning since they indicate various properties of helenine. The acetone precipitate was used as starting material unless otherwise stated.

Cetyl pyridinium chloride (CPC) precipitated helenine from water solutions. The CPC-helenine complex was dissociated by dissolving it in ethanol, a process which precipitated the helenine and dissolved the CPC. A five-fold purification was obtained by this method.

Treatment with 5% trichloroacetic acid caused complete loss of activity, probably because of the low *p*H. Ammonium sulfate was ineffective in

(15) A. Tissières and J. D. Watson, *Nature*, **182**, 778 (1958).

(16) H. T. Shigeura and E. Chargaff, *Biochim. et Biophys. Acta*, in press.

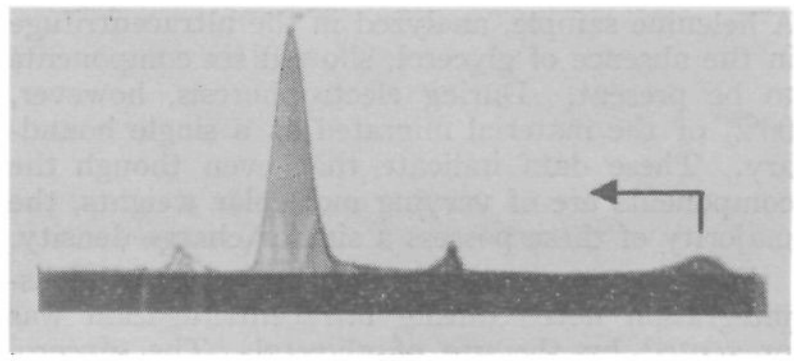

Fig. 4.—Electrophoresis pattern of helenine purified by ultracentrifugation. The buffer was 0.004 *M* phosphate, *p*H 7, containing 0.005 *M* Mg^{++}. The descending pattern is shown after 85 min. at 19.3 v./cm. The mobility of the major peak was -4.8×10^{-5} cm.²/sec./v.

precipitating material even at 90% of saturation. This was very likely due to the low concentration of helenine in the acetone precipitate. The method might prove useful when applied to more highly purified material.

Fractionation with acetone produced an enrichment. Some inactive material was precipitated at an acetone concentration of 25%, after which the active component came down at slightly higher acetone concentrations. All the helenine had been precipitated at an acetone concentration of 50%, leaving behind considerable acetone-soluble material. Purifications of two- to three-fold could be obtained.

Calcium phosphate gel was a very effective adsorbent for helenine. The activity could be adsorbed from a solution at *p*H 5 and eluted from the gel by increasing the *p*H to 8. The gel could be used either batchwise or in a column.

A number of ion-exchange resins with varying degrees of crosslinking were tried, but helenine was never successfully adsorbed.

Discussion

As was detailed above, highly purified helenine was obtained by extraction of the mycelium with dilute neutral buffer containing Mg^{++} and glycerol, precipitation of the active component with acetone, ultracentrifugation and finally chromatography of the sedimented material on ECTEOLA-cellulose.

Preparations obtained in this manner have been found by chemical tests to be essentially nucleoprotein. Unfortunately final characterization of helenine as a member of this class of compounds cannot be made because of the heterogeneity of the material. The best preparations obtained have shown two major components and one minor one during ultracentrifugation. It seems likely, though, that the observed inhomogeneity is an inherent property of the material and one that is common to nucleoproteins. Tissières and Watson,[15] working with nucleoprotein from *Escherichia coli*, noted the presence of two main components with $S_{20,w}$ values of 70 and 100 *S*, the 100 *S* material being present in the largest quantity. By alteration of the buffer medium, the molecule dissociated into smaller components. This is essentially what was observed with highly purified helenine.

Additional evidence for the dissociation theory was obtained from data shown in Figs. 2 and 4.

A helenine sample, analyzed in the ultracentrifuge in the absence of glycerol, showed six components to be present. During electrophoresis, however, 90% of the material migrated as a single boundary. These data indicate that even though the components are of varying molecular weights, the majority of these possess a similar charge density.

Furthermore, as seen in Fig. 2A, most of the disintegration noted during ultracentrifugation was prevented by the use of glycerol. The glycerol made it possible to obtain a preparation that exhibited only two major peaks and one minor one.

The failure of the bacterial endotoxins and the lipopolysaccharide fraction from *P. funiculosum* to produce a helenine-like response in the assay suggests that helenine is different from these compounds. The low hexose content of our purified nucleoprotein fraction would also support this view.

Acknowledgment.—We wish to thank Miss Verda Powell and Mr. John Ruscica for many of the electrophoresis and ultracentrifuge determinations. We would also like to acknowledge the excellent technical assistance of Mrs. Elizabeth Hagan.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE RICE INSTITUTE, HOUSTON, TEX.]

Observations on the Structure of Oxonitine[1]

BY RICHARD B. TURNER, J. P. JESCHKE AND M. S. GIBSON

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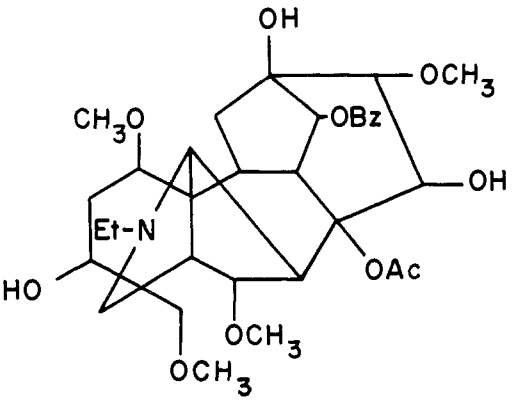
Conclusive evidence has been obtained in support of the contention of Pelletier that oxonitine, an oxidation product of aconitine, is an N-formyl derivative (IV). That the formyl group is not derived from the N-ethyl group of aconitine (for example, by oxidative cleavage of a vinylamine) is indicated by the results of radioactive tracer experiments. The N-acetyl derivative II has also been isolated as a minor product of the permanganate oxidation of aconitine, and cleavage of N-nitrosodesethylaconitine triacetate (VIII) by acetyl chloride and by phosgene has been demonstrated.

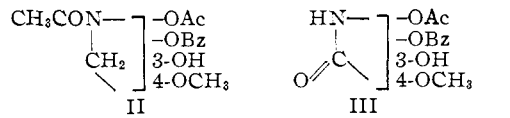
Early investigation of the Aconite alkaloid aconitine showed that this substance is a tertiary base of molecular formula $C_{34}H_{47}NO_{11}$ possessing an N-ethyl group, four methoxyl groups, three hydroxyl groups, one acetoxyl group and one benzoyloxy group.[2] The problem of skeletal structure proved exceedingly difficult, and little progress was made in its solution until 1956, when the structure of the closely related alkaloid, lycoctonine, was deduced by Przybylska and Marion from X-ray measurements.[3] The recent results of Wiesner, Büchi and their collaborators[4] now provide strong evidence in support of structure I for aconitine.

In the historical development of the aconitine work the permanganate oxidation product, oxonitine, occupied a position of considerable importance. Oxonitine possesses an amide linkage and gives negative results in the Herzig–Meyer determination indicating loss of the original N-ethyl group.[5] With the exception of this modification the functional groups of aconitine are present intact in the oxidation product. However, despite repeated investigation, the nature of the structural change involved in the formation of oxonitine has remained in doubt.



I

The uncertainty regarding the formulation of oxonitine has centered mainly on the two part structures II and III which correspond, respectively, to the molecular formulas $C_{34}H_{45}NO_{12}$ and $C_{32}H_{41}NO_{12}$. Although these alternatives should be easily distinguishable by elementary analyses,



considerable difficulty has been experienced with aconitine derivatives in the preparation of pure, solvent-free analytical samples.[6] Thus, Jacobs and Pelletier[7] report that oxonitine crystallized from chloroform contains a substantial amount of chlorine owing to tenacious retention of solvent. Acetic acid is also objectionable. While a series of samples giving carbon and hydrogen values corresponding closely to those calculated for II were

(1) This investigation was supported by a grant from the Robert A. Welch Foundation.

(2) *Cf.* K. Wiesner and Z. Valenta, "Progress in the Chemistry of Organic Natural Products," L. Zechmeister, editor, Vol. XVI, Springer-Verlag, Vienna, 1958, pp. 26–89; E. Stern, Chap. 37 in "The Alkaloids, Chemistry and Physiology," R. H. F. Manske and H. L. Holmes, editors, Vol. IV, Academic Press, Inc., New York, N. Y., 1954, pp. 275–333; T. A Henry, "The Plant Alkaloids," J. A. Churchill, London, 1949, pp. 674–678.

(3) M Przybylska and L. Marion, *Can. J. Chem.*, **34**, 185 (1956).

(4) K. Wiesner, M. Götz, D. L. Simmons, L. R. Fowler, F. W. Bachelor, R. F. C. Brown and G. Büchi, *Tetrahedron Letters*, No. 2, 15 (1959); K. Wiesner, F. Bickelhaupt, D. R. Babin and M. Götz, *ibid.*, No. 3, 11 (1959); K. Wiesner, D. L. Simmons and L. R. Fowler, *ibid.*, No. 18, 1 (1959); see also M. Przybylska and L. Marion, *Can. J. Chem.*, **37**, 1116, 1843 (1959).

(5) W. A. Jacobs and R. C. Elderfield, THIS JOURNAL, **58**, 1059 (1936).

(6) *Cf.* E. Späth and Fr. Galinovsky, *Ber.*, **63**, 2994 (1930).

(7) W. A. Jacobs and S. W. Pelletier, THIS JOURNAL, **76**, 4049 (1954).