Although we could not find any relation between complement and vitamin C, we do not wish to draw any conclusions as to the possible influence of the vitamin on other immunological processes. The exact significance of complement in immunology is in any case still uncertain.

SUMMARY

1. Complement titrations, using 50 % haemolysis as the end-point, were performed on forty guinea-pigs maintained on different levels of intake of vitamin C, and the results were evaluated statistically.

2. No significant change in complement was

found to occur in guinea-pigs partially or completely deficient in vitamin C.

3. Addition of cabbage to the diet did not result in any change in the complement titre as compared with that of guinea-pigs whose diet was supplemented with adequate amounts of synthetic vitamin C.

4. The significance of these results is discussed.

We wish to thank Dr L. J. Harris, at whose suggestion this work was undertaken, for his valuable advice and interest throughout the experiment, Prof. H. R. Dean for his interest and facilities for work in his Department for one of us, Dr J. O. Irwin for his criticism of the statistical calculations, and Mr S. Impey for his help in the care of the experimental animals.

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Antigenic Properties of Hyaluronic Acid

By J. H. HUMPHREY (Jenner Memorial Student), *The Lister Institute of Preventive Medicine, Elstree, Herts*

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Hyaluronic acid has been shown by Meyer and colleagues [Meyer & Palmer, 1936; Meyer, Smyth & Dawson, 1939; Meyer & Chaffee, 1941] to occur in the vitreous humour, synovial fluid, skin and umbilical cords of various mammals and by the author (unpublished) to occur in the lung tissue. Although the detailed structure of this substance has not been worked out, the work of Meyer and his collaborators [reviewed by Meyer, 1938] has shown hyaluronic acid to be a polysaccharide of high molecular weight, composed of units containing one residue of N-acetyl glucosamine linked with one of glucuronic acid. Existing evidence suggests that specimens of hyaluronic acid prepared from different

sources are chemically identical. A polysaccharide which is probably identical has been obtained from capsulated streptococci of groups A and C by Kendall, Heidelberger & Dawson [1937] and by Seastone [1939]. The capsules of these organisms are destroyed by an enzyme—hyaluronidase—obtained from other strains of streptococci or from various other sources [McClellan, 1941]. Their presence is associated with the virulent stage of the organisms, but when either whole organisms or purified capsular material were injected into rabbits they failed to give rise to detectable circulating antibodies against the capsules [Seastone, 1939].

In view of these results it seemed of interest to investigate more closely the antigenicity of hyaluronic acid, since not only would specific antisera to this substance be of diagnostic value and possibly of therapeutic use, but the question of antibody production in this instance has a general bearing on theories of antibody formation. It was known (unpublished information from Dr W. T. J. Morgan), that combination of hyaluronic acid with the specific protein obtained from *Shigella dysenteriae* by Morgan & Partridge [1940] was ineffective in conferring antigenic properties, although with other polysaccharides [Morgan & Partridge, 1940; Partridge & Morgan, 1940, 1942] this method is very successful. Recourse was therefore had to the method used by Goebel & Avery [1931] in their work on the artificial coupling of type III *Pneumococcus* polysaccharide to protein by means of a *p*-aminobenzyl ether linkage. The method of Goebel & Avery was not directly applicable to hyaluronic acid since it was found that the number of *p*-nitrobenzyl ether groups introduced varied with the proportion of *p*-nitrobenzyl bromide to hyaluronic acid used, and the different products had very varied solubilities in H_2O and in organic solvents. It may be assumed that the product containing the minimum number of introduced groups is to be preferred, provided that there are enough for effective coupling, since extra groups may mask or modify a weakly antigenic grouping in addition to conferring their own antigenic specificity. A substance has now been prepared containing approximately one *p*-aminobenzyl group per glucosamine residue. It was coupled to horse serum albumin and to normal rabbit serum, the former being used for immunizing and the latter as test antigen, thereby eliminating the effects of non-specific antibodies.

EXPERIMENTAL

(a) Chemical

Hyaluronic acid. The starting material was the potassium salt of hyaluronic acid prepared from human umbilical cords, purified by repeated extraction with 90% phenol solution and by repeated precipitation from aqueous solution at pH 9–10 by $1\frac{1}{2}$ vol. of ethanol saturated with K acetate. It contained only 75% of the theoretical glucosamine content as determined by Hewitt's modification [Hewitt, 1938] of the method of Morgan and Elson, and was contaminated with finely divided insoluble haematin derivatives extracted with the hyaluronic acid from the cords. These impurities were very difficult to separate from the highly viscous hyaluronic acid without destroying its viscosity. They were, however, readily removed by centrifugation after treatment with *p*-nitrobenzyl bromide as shown below. (Later this work was repeated on a smaller scale using the pure K salts of hyaluronic acid as starting material, with similar results.)

Aminobenzyl ether of hyaluronic acid. To 150 ml. of a 1% solution of hyaluronic acid was added 0.8 g. of finely

powdered *p*-nitrobenzyl bromide. The mixture was heated to 100° on a water-bath and 0.75 ml. 20% NaOH added drop-wise and with vigorous stirring during a period of 15 min. Heating was continued for a further 30 min. and the material then neutralized with a drop of acid, steam-distilled to remove traces of *p*-nitrobenzyl alcohol, and centrifuged to remove insoluble matter. The clear solution was dialysed against 3 l. of distilled H_2O and was finally dried *in vacuo* from the frozen state, to give 0.7 g. white, very hygroscopic material. Analysis on dry basis: N=4.65%; glucosamine=38.5%; glucosamine/N ratio=8.4; ash negligible. $[\alpha]_D^{20} = -58^\circ$. Viscosity of a 1% solution in a crude Ostwald viscosimeter was $9.5 \times H_2O$. (Theory for mono-nitrobenzyl derivative of hyaluronic acid: N=5.25%; glucosamine=33.6%; glucosamine/N ratio=6.4.)

The solution of the *p*-nitrobenzyl ether was reduced with saturated $Na_2S_2O_4$ solution at 50°, the reaction being maintained at approximately pH 7, more $Na_2S_2O_4$ being added until the solution bleached litmus paper. It was then dialysed against distilled H_2O until the dialysate gave no yellow colour with Ehrlich's reagent, indicating that the diffusible amino-groups had been removed. The contents of the dialysis bag still gave a definite yellow colour with Ehrlich's reagent. The solution was evaporated to small volume *in vacuo*, and dried *in vacuo* from the frozen state to yield 0.6 g. of a very pale yellow hygroscopic aminobenzyl ether of hyaluronic acid. Two samples prepared independently from separate lots of hyaluronic acid gave the following analyses on a dry basis. *Sample 1*: N=4.75%; glucosamine=40%; glucosamine/N ratio=8.4; $[\alpha]_D^{20} = -40^\circ$; viscosity of 1% solution= $8.5 \times H_2O$. *Sample 2*: N=5.00%; glucosamine=40%; glucosamine/N ratio=8.1; $[\alpha]_D^{20} = -65^\circ$; viscosity of 1% solution= $8.6 \times H_2O$.

Theory for mono-aminobenzyl ether of hyaluronic acid is N=5.4%; glucosamine=34.5%; glucosamine/N ratio=6.4. These analyses, although in fair agreement with one another, do not exactly conform to those expected if one aminobenzyl group is coupled to one hyaluronic acid unit. A compound containing 0.66 aminobenzyl groups per hyaluronic acid unit could theoretically give the following analysis: N=4.9%; glucosamine=37.5%; glucosamine/N ratio=7.7, which is in close agreement with the figures obtained. In view of the present uncertainty concerning the detailed structure of hyaluronic acid no reason can be given why, under the conditions used, there should be formed a compound which appears to consist of two aminobenzyl groups per three hyaluronic acid units, nor even whether it is a definite compound. No additional information can be obtained from values for the specific rotation, as these vary from preparation to preparation of hyaluronic acid.

The compound was acted on by testicular hyaluronidase to yield a substance giving the colour reaction for N-acetyl glucosamine. In 1% solution it gave also a definite yellow colour with Ehrlich reagent, and after diazotization followed by removal of excess HNO_2 with ammonium sulphamate, a good pink colour was given with a 0.5% solution of N-1-naphthyl ethylenediamine, indicating the presence of an aromatic NH_2 group. In order to rule out the possibility that the yellow colour given with Ehrlich's reagent might be due to a mixture of unchanged hyaluronic acid and traces of *p*-aminobenzyl alcohol, the substance was extracted with acetone, dioxan and glacial acetic acid without, however, removing any substances giving the Ehrlich colour reaction.

Coupling of the aminobenzyl ether of hyaluronic acid to protein. On the assumption of 1 amino-group per glucosamine residue, 0.5 g. of the aminobenzyl ether of hyaluronic acid preparation was diazotized at 0° with 2.2 equivalents of *N* HCl and 1.0 equivalent of *N* NaNO₂. Stirring was continued at 0° for 30 min. and the solutions carefully neutralized to phenol red. The solution gave a strong red colour with alcoholic β -naphthol.

The proteins used were horse-serum albumin and whole-rabbit serum, in the proportions of 3.6 g. protein (calculated from the N content) to 1 g. of the compound. The protein solution (*ca.* 2%) was kept chilled and stirred, and the diazotized compound was added slowly, together with sufficient NaOH to maintain the solution just alkaline to phenolphthalein. Stirring was continued for 1 hr. and sufficient 10% trichloroacetic acid then added to give maximum precipitation. The precipitate was spun down, separated and redissolved in H₂O with a minimum of NaOH. After two or three reprecipitations the supernatant was no longer yellow. The coupled protein was finally made up to about 2% in 0.9% NaCl and dialysed against 0.9% NaCl until it was free from CCl₃COOH. A similar procedure was used in coupling with rabbit serum, but here precipitations of the coupled protein with CCl₃COOH proved to be incomplete and uncoupled material was removed by prolonged dialysis against 0.9% NaCl.

The final products gave pale yellow solutions, and different preparations contained from 5 to 9% glucosamine, calculated from the glucosamine/N ratio. The original serum albumin contained 1.5% glucosamine, which suggests that 7.5–17% of hyaluronic acid had been added by the coupling.

(b) Serological

Immunization. Three rabbits were immunized with the horse serum-hyaluronic acid preparation. The first course consisted of 30, 60 and 120 mg. injected subcutaneously at weekly intervals. After a week's rest the animals were bled. The second course consisted of four intravenous injections of 20 mg. at weekly intervals, and the animals were bled out 10 days later. None of the animals showed any ill effects during immunization, nor any changes post-mortem such as might indicate antibody formation against a common constituent of the tissues.

Technique of tests on antisera. Precipitin tests were carried out in bulk using constant serum (1:5 dilution) and fivefold dilutions of antigen.

Complement fixation was carried out using two minimal haemolytic doses (M.H.D.) of complement, and the same concentrations of serum and antigen as in the precipitin tests.

Agglutination was tested on a washed suspension of cells from a young culture of a capsulated group C *Streptococcus* whose capsules were known to be dissolved by hyaluronidase. Capsular swelling was tested by incubating equal volumes of serum and streptococcal suspension at 37° for 10 min. and then viewing in wet preparation against a background of India ink.

RESULTS

The results with the serum obtained after the first and after the second course of immunization were substantially the same. Each serum was tested

separately, but all behaved similarly. The results are shown in Table 1.

Table 1. *Serological tests on sera of rabbits immunized with hyaluronic acid coupled with horse-serum albumin, against various test antigens*

Test antigen	Precipitin titre	Complement fixation titre
Horse-serum albumin coupled with hyaluronic acid	1:10,000	1:1000
Whole rabbit serum protein coupled with hyaluronic acid	Nil	Nil
Normal horse-serum albumin	1:10,000	1:1000
Normal whole rabbit serum protein	Nil	Nil

The positive reactions are evidently due to antibodies against the horse-serum moiety of the antigen, and not against the specific coupled hapten group. No attempt was made to see whether the uncoupled substances would inhibit the reaction between the coupled proteins and homologous antisera. There was no evidence of agglutination nor of capsular swelling in the tests with *Streptococci*.

DISCUSSION

The retention of viscous properties and the hydrolysis by testicular hyaluronidase argue that the structure had not been much altered, and yet it is clear from the above results that even when chemically coupled to a protein, hyaluronic acid is unable to cause antibody formation in rabbits. In this it differs from various chemically similar muco-polysaccharides such as those from pneumococci, from *Shigella dysenteriae*, or the blood group 'A' antigen, but resembles chondroitin sulphuric acid and glyco-gen, which are also common natural constituents of the mammalian body. The reason for this difference is unexplained. There may be chemical considerations which make the former class of substance able to influence immune serum protein production, while the latter class is unable, but it seems easier to suppose that the second class are of such wide distribution in mammalian tissue that they occur naturally at the sites of antibody production, and therefore exert no different or additional influence when supplied by artificial means.

SUMMARY

Hyaluronic acid coupled chemically to protein by azobenzyl ether linkage does not act as a specific hapten when injected into rabbits.

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The Excretion of Ethylquinone by the Flour Beetle

By P. ALEXANDER AND D. H. R. BARTON, *Department of Inorganic and Physical Chemistry, Imperial College, London*

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While studying inert dust insecticides we observed that the red rust flour beetle (*Tribolium castaneum*) made a brown stain when kept in contact with various fine white powders such as amorphous precipitated silica. When placed on these dusts and kept in dry air the insects did not move about in the material after the first few hours and died after about 2 days. The brown stain developed uniformly round the insect and became visible after the first day, but did not reach its maximum strength until about the sixth day, i.e. long after the insect had died. A similar but smaller stain was produced when dead insects were placed on the powder.

The closely related insect, *Tribolium confusum*, is known to give off an excretion which causes heavily infested flour to become pink and quite unpalatable, and which Payne [1925] found to exhibit aldehyde reactions. Chapman [1926] observed that the same beetle, when stimulated, gave off a nose-irritant gas which turned flour pink. He examined the effect of this gas on the pupae of the beetle and found that the subsequently metamorphosed adults were abnormal. Roth & Howland [1941] passed dry air over *T. confusum*, and isolated the excretion as yellow-brown needles which turned red and eventually blue, even when kept in solid CO₂. At room temperature a gas was evolved of quinone-like odour which was capable of oxidizing HI to I₂. These authors found that the last instar larvae, prepupae and pupae of the insect all metamorphosed into abnormal adults after exposure to this gas.

EXPERIMENTAL

Isolation of the excretion from T. castaneum

The method used for isolating the excretion from *T. castaneum*, based on that used by Roth & Howland [1941] for *T. confusum*, was as follows: About 500–1000 insects (a

volume of about 5 ml.) were collected from the insect cultures by taking advantage of the negative geotropism of the adult beetles, sifted free from flour and introduced into a small distilling flask. A stream of dry air was then passed through the flask for about 6 hr., during which time the insects were cooled from 35–40 to 0° for about 20 min. every 2 hr. The excretion was condensed in an ethanol-CO₂ trap, yielding about 0.5 mg. of long, needle-like yellow crystals together with some ice, from the H₂O also given off by the insects. The yield was considerably reduced if the insects were not completely free from flour and larvae, etc. The insects were returned to flour for 3 days and then the procedure was repeated, giving a somewhat smaller yield. After three or four such operations the insects were very feeble and yielded little or no excretion. Many were dead, due, probably, to desiccation. Attempts to separate the H₂O from the excretion by drying with P₂O₅, CaCl₂, Na₂SO₄ or Mg(ClO₄)₂ yielded a reddish 'polymer' of the excretion on the surface of the drying agent, even when the drying tubes were warmed to 40°.

In view of the small yield per insect (about 0.1–0.2% of the initial body weight) the excretion from some 5000 beetles was necessary for the investigation outlined below.

RESULTS

Properties of the excretion from T. castaneum

The yellow crystals, separated from the H₂O on a cooled porous tile, had a nose-irritant quinone-like odour and were slightly lachrymatory. They were slightly soluble in H₂O, but readily so in ethanol, benzene, chloroform and other organic solvents. The substance was fairly volatile at room temperature and rather labile when first isolated, turning to a red-brown on standing overnight, even at 0°. However, drying increased the stability. The aqueous solution was neutral, reduced Tollen's reagent, liberated I₂ from HI, and gave a brown-black colour with warm dilute alkali. The substance was very sensitive to alkali, but comparatively stable to acid.