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The Aspermatogenic Antigen in Experimental Allergic Orchitis in Guinea-Pigs

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Summary. Purification of the aspermatogenic factor of guinea-pig testes has resulted in an antigen capable of inducing circulating antibody, delayed skin reactivity and a testis lesion on injection of as little as 5 μg into guinea-pigs.

Chemical analysis of the fraction revealed a nitrogen content of 7·7 per cent and a polysaccharide content of 38 per cent.

INTRODUCTION

Aspermatogenesis was first produced in guinea-pigs by Freund, Thompson and Lipton (1955) by subcutaneous injection of a water-in-oil emulsion of guinea-pig testicular tissue containing killed mycobacteria. Freund *et al.* concluded that aspermatogenesis resulted from immunological injury to the testis, and showed that considerable purification of an active antigenic aspermatogenic factor was achieved by removal of protein from an extract of testicular tissue by consecutive precipitation with ammonium sulphate, trichloracetic acid and chloroform–butyl alcohol mixture. Their purified fraction was at least a 100 times more potent in inducing aspermatogenesis than the original extract.

We have previously described (Brown, Glynn and Holborow, 1963) the use of phenol extraction and papain digestion to obtain active aspermatogenic material from autoclaved testis products. In the present paper we report the separation and chemical analysis of a fraction aspermatogenic in doses as low as 5 μg.

MATERIALS AND METHODS

Fractionation procedure

This is shown in Fig. 1. The testis was homogenized and autoclaved three times. The pooled extracts were treated with phenol saturated with water and the material in the phenol phase was recovered by dialysis and digested with papain before re-extraction with phenol. The water phase resulting from this extraction was then treated with trichloracetic acid.

The autoclaving, papain treatment, trichloracetic acid treatment and the second phenol extraction were carried out by the methods previously described (Brown *et al.*, 1963). The first phenol extraction was carried out in the presence of 3 per cent sodium chloride.

Between each treatment the fractions were dialysed against distilled water and freeze-dried.

Immunization procedure and skin tests.

The methods used were the same as those described by Brown *et al.* (1963).

Fluorescent antibody technique

Tissues. Normal guinea-pig testes were fixed by the cold alcohol method and the sections prepared from paraffin blocks as described by Sainte-Marie (1962).

Fluorescent staining. The sandwich method was used, where the sections are first treated with the antiserum and then stained with a conjugate prepared as previously described (Brown *et al.*, 1963) but containing the non-specific counter-stain rhodamine-labelled bovine serum-albumin described by Smith, Marshall and Eveland (1959).

Chemical estimations

Nitrogen was estimated by the micro-Kjeldahl method using Fawcett's digestion procedure (1954).

DNA. An approximate value was obtained by reading the optical density at a wavelength of 257·5 mμ giving an average of the purines and pyrimidines present expressed as DNA.

Hexosamine was estimated by the method of Elson and Morgan (1933).

Reducing sugars. The cysteine hydrochloride method of Dische (1962) was used. Approximate proportions of mannose, galactose and glucose were estimated by leaving the tubes overnight and reading the resulting colour changes at a wavelength of 605 mμ.

Uronic acid was estimated by the carbazole method of Dische (1946).

Sialic acid. The direct Ehrlich method of Barry, Abbot and Tsai (1962) was used.

Amino-acid chromatography

A two-way chromatogram was run using first, butanol–acetic in one direction, and secondly metacresol ammonia in the other. The spots were stained with ninhydrin. Hydrolysis was in 6 N HCl at 100° overnight.

Sugar chromatography

A one-way chromatogram was run in butanol–acetic and stained with aniline hydrogen phthalate. Hydrolysis of the extract was in 1 N HCl for 3 hours at 100°.

RESULTS

ACTIVITY OF THE TESTIS FRACTIONS

Fig. 1 shows that on phenol extraction of whole testis autoclavate, the aspermatogenic activity appeared in the phenol-soluble fraction. After papain digestion of this phenol soluble material, however, further extraction with phenol resulted in separation of aspermatogenic activity in the water-phase. When this water phase was treated with trichloracetic acid the antigen remained soluble.

This last fraction, when injected in doses as low as 5 μg with complete adjuvant intradermally into guinea-pigs gave, 4 weeks later, circulating antibody, delayed skin reactions and a testis lesion. When whole testis homogenate is used, a dose of 10 mg is necessary to produce the same type of lesion in the same proportion of guinea-pigs.

The histology of the lesion was similar to that previously described (Brown *et al.*, 1963). There was extensive atrophy of the seminiferous tubules with only one layer of basal cells remaining in the majority of the tubules. Cellular infiltration was, as before, sometimes present and sometimes absent.

The presence of circulating antibody was shown by the fluorescent antibody method and the type of staining obtained with antibody to the purified fraction was the same as that obtained using antiserum to whole testis homogenate. The staining was confined to the cytoplasm of groups of cells in the central area of the tubules, probably the secondary spermatocytes, spermatids and sperms. When the cells were examined under the oil immersion ($\times 1000$) not all the cytoplasm was stained, but a cap-like area resembling the shape of the acrosome. When individual sperms were stained, only the acrosomes stained positively.

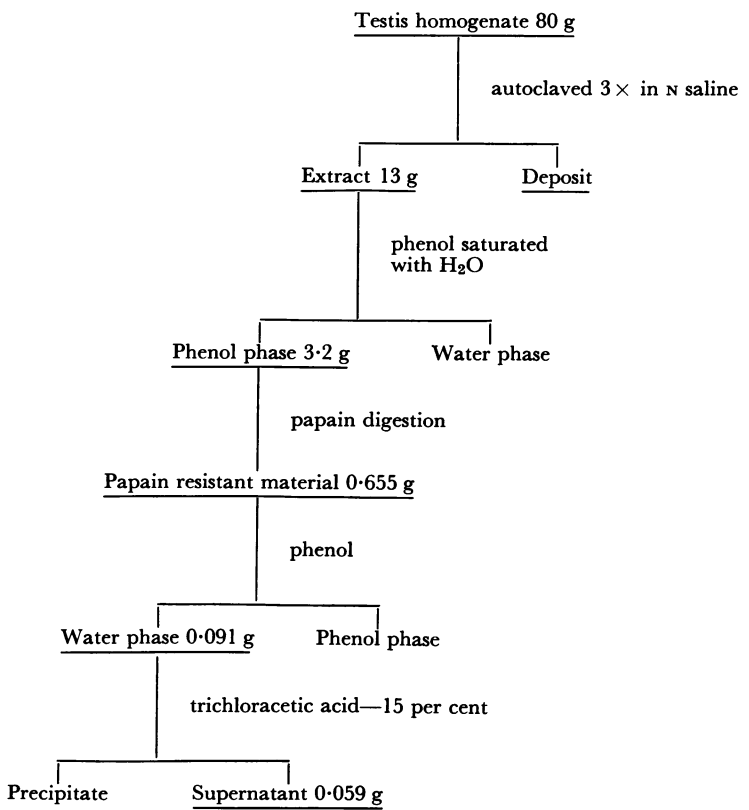

FIG. 1. Fractionation of testis homogenate (aspermatogenic fractions are underlined).

CHEMICAL ANALYSIS OF THE TESTIS FRACTIONS

The results of the analysis are listed in Table 1. It is seen that a large proportion of the DNA in the autoclavate was removed in the non-active water phase of the first phenol treatment. There was some decrease in the nitrogen concentration during the fractionation but the purified fraction still contained 7·7 per cent.

The greatest changes achieved by the fractionation were in the hexosamine and reducing sugar concentrations, both rising with each successive step. The hexosamine concentration rose from 2·2 per cent in the first phenol extract to 16·7 per cent in the purified fraction and the reducing sugars from 1 per cent to 11·5 per cent expressed as

TABLE 1

CHEMICAL ANALYSIS OF TESTIS FRACTIONS

	N_2 (%)	DNA (%)	Hexosamine (%)	Reducing sugars (%)		Sialic acid (%)	Uronic acid
				As glucose	As galactose		
Testis autoclavate							
Water phase	6·0	48	—	—	—	—	—
Phenol phase	12·1	5·2	2·2	1	1·96	—	—
papain							
Papain resistance material	12·4	11·2	6·5	3·1	6·2	—	—
phenol							
Phenol phase	8·1	11·8	9·0	7·6	13	—	—
Water phase	8·4	7·6	12·5	6·5	12·5	—	—
TCA							
Supernatant	7·7	4·6	16·7	11·5*	20*	6	0

*Probable proportions 9 per cent galactose and mannose, 6 per cent glucose.

glucose, or from 2 per cent to 20 per cent expressed as galactose. The probable concentration being 15 per cent; 9 per cent of galactose and mannose and 6 per cent of glucose. There was also a sialic acid concentration of 6 per cent.

Although the uronic acid estimation was unsatisfactory owing to the very high readings obtained with the tissue blanks, there was probably none present in the purer fraction.

Amino acid chromatography (Fig. 2) revealed the presence of various amino acids. Strongly staining spots occurred for aspartic and glutamic acids, serine, threonine and lysine; medium strong for glycine, alanine and proline; medium for valine, phenylalanine, leucine and glucosamine and weakly staining for arginine. There was no tyrosine present.

Chromatography for sugars revealed the presence of galactose, glucose and a weak spot for mannose.

DISCUSSION

Despite the high biological activity of the isolated fraction there is as yet no conclusive evidence that it is a single substance. The DNA is almost certainly a contaminant; the figure of 4·6 per cent represents a maximum estimate, since some of the ultraviolet absorption must be due to degraded products of nucleic acids. The remainder has the composition of a glycoprotein with about 48 per cent peptide and 38 per cent polysaccharide. About 10 per cent remains unaccounted for. In the autoclaved material the

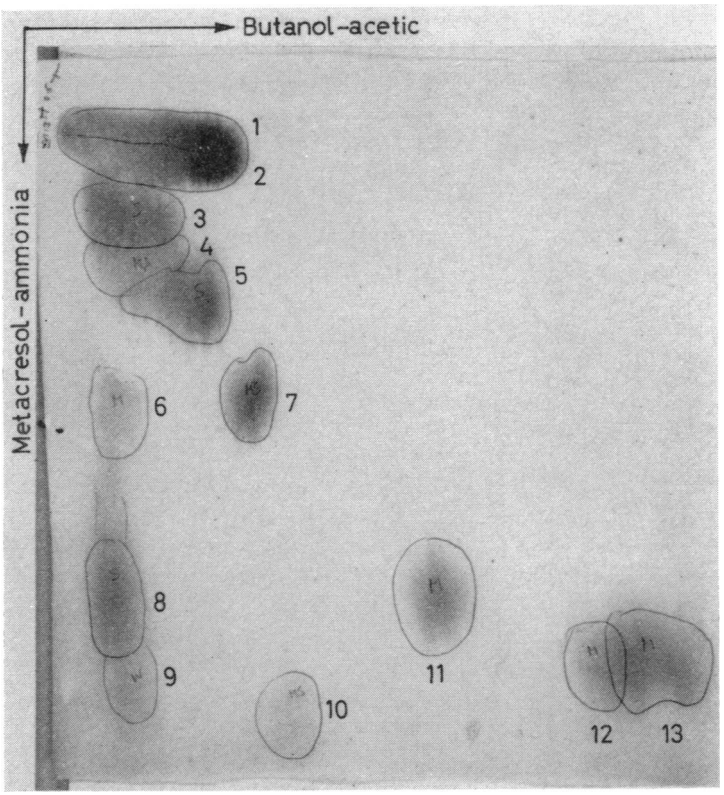

FIG. 2. Two-dimensional separation of the purified fraction by paper chromatography. 1, Aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, threonine; 6, glucosamine; 7, alanine; 8, lysine; 9, arginine; 10, proline; 11, valine; 12, phenylalanine; 13, leucine.

peptide content of the active factor is probably much higher, which would account for its extraction into the phenol phase. Part of this peptide is presumably removed by papain so that after such treatment the activity on phenol extraction remains in the aqueous phase. At this stage therefore the material behaves like one of the blood group substances. The similarity also in amino acid composition is remarkable. All the eleven amino acids found by Morgan and his colleagues (Annison and Morgan, 1952a, b; Gibbons and Morgan, 1954) in the blood group substances obtained from human ovarian cyst fluid are present; the only additional acid we have found is phenylalanine. Tyrosine is absent from both. The sugar components are however entirely different, the absence of galactosamine and of fucose and the presence of sialic acid being the most obvious features.

By analogy with blood group substance one would expect the aspermatogenic factor to elicit as antigen both circulating antibody and delayed hypersensitivity, the specificity of the former determined by the polysaccharide and that of the latter by the peptide moieties (Holborow and Loewi, 1962). The purified highly active fraction described here produces aspermatogenesis in the presence of both circulating antibody reactive with acrosomal material of testis cells, and delayed hypersensitivity to this fraction itself. We have previously described the inhibition of aspermatogenesis and delayed

hypersensitivity, although not of circulating antibody, by prior immunization with testis material in incomplete adjuvant (Brown *et al.*, 1963) and a similar observation has been made by Chutna and Rychlikova (1964). We are now investigating this effect using the purified aspermatogenic fraction.

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