THE ISOLATION OF A SPECIFIC SUBSTANCE AND A GLYCOGEN-LIKE POLYSACCHARIDE FROM *TRICHOMONAS FOETUS* (VAR. MANLEY).

J. G. FEINBERG AND W. T. J. MORGAN.

*From the Lister Institute of Preventive Medicine, London, S.W.*1.

Received for publication October 20, 1952.

Trichomonas foetus is a protozoan flagellate which inhabits the genital tract of bovines and is responsible for much of the abortion and infertility in this species. In the bull it inhabits the preputial cavity, from which it is transmitted to the cow by coitus and subsequently proliferates in the uterus.

Kerr and Robertson (1945) distinguished two varieties of the organism on the basis of immunological behaviour in bovines and laboratory animals and designated the varieties " Manley " and " Belfast."

Svec (1944) attempted to isolate specific substances from *T. foetus*, using a modification of the method introduced by Fuller (1938) for the isolation of specific carbohydrates from streptococci. The culture was extracted with hot formamide and the soluble material separated into several fractions by the addition of alcohol. The carbohydrate isolated failed to induce a skin reaction in artificially immunized cows, and at high concentration gave only poor precipitation with bovine immune *T. foetus* anti-sera. These fractions and other preparations were examined further by B. B. Morgan (1948) in an unsuccessful attempt to elicit skin reactions in cattle and obtain precipitation reactions with the serum of rabbits and cattle immunized with *T. foetus* cultures. MacDonald and Tatum (1948) also reported they were unable to obtain a specific precipitation with these preparations and the serum of rabbits inoculated either with the supernatant fluid from old cultures of *T. foetus* or with materials obtained by the extraction of organisms with formamide or as a result of sonic disruption.

The present paper describes the isolation of a material from *T. foetus* which possesses the same dominant serological specificity as the intact protozoon. The specific substance is extracted from the organism with anhydrous diethylene glycol, a procedure already extensively developed (Morgan, 1937 ; Henderson and Morgan, 1938 ; Morgan and Partridge, 1940, 1941 ; Partridge and Morgan, 1942 ; Morgan, 1949) for the extraction of specific antigenic materials from certain organisms of the genera *Shigella* and *Salmonella*. The isolation of a serologically inactive polysaccharide, most probably glycogen, from *T. foetus* is also described.

MATERIALS AND METHODS.

Trichomonas foetus.—The organisms were grown in pure culture for 48 hr. at 37° in 1 l. quantities in a glucose serum broth medium (Kerr and Robertson, 1947) to reach a density of about 5×10^6 cells per ml. The organisms were collected and washed with sterile saline by centrifugation and immediately dried from the frozen state. About 0·5 g. dry organisms was obtained from each litre of culture fluid.

Organic solvents.—Commercial ethylene glycol (b.p. 197°) and diethylene glycol (b.p. 245°) were re-distilled and only the constant boiling material was collected for use.

Agglutination tests.—Performed by the method of Robertson (1941) using Petri dishes to observe agglutination. A capillary agglutination test introduced by Feinberg (1952) was also used.

Precipitation tests.—A capillary precipitation test similar to that described by Dubos (1948) for typing streptococci was used with certain modifications.

Nitrogen content.—Determined by the Kjeldahl method using the apparatus of Markham (1942) and a mixed bromocresol green and methyl red indicator (Ma and Zuazaga, 1942).

Phosphorus.—Estimated by the method of Martland and Robison (1926).

Reducing power.—Estimated by the method of Somogyi (1937).

Hexosamine.—Determined by a modification of the method of Elson and Morgan (1933) using glucosamine concentrations of 10–40 μg. as standards, a photoelectric colorimeter and a green light filter (λ, 540 mμ.).

Rhamnose.—Estimated by the method of Dische and Shettles (1948).

Chromatographic methods.—Paper chromatography as developed by Consden, Gordon and Martin (1944), using as solvents collidine, phenol and *n*-butanol and Whatman No. 1 paper, was used for the qualitative examination of the amino-acids, hexosamines and sugars. The amino-acids were detected by spraying the papers with ninhydrin. The sugar spots were developed by spraying with $AgNO_3/NH_3$ and heating to 105° for 5 min. (Partridge, 1946), or by treatment with aniline hydrogen phthalate (Partridge, 1949). The hexosamine was detected on the paper sheets or strips as cherry red areas, by spraying with an alkaline acetylacetone reagent followed by *p*-dimethylaminobenzaldehyde in HCl-*n*-butanol (Partridge, 1948).

Isolation of the Specific Substance.

The freeze-dried organisms were extracted successively with alcohol, ether, acetone, chloroform and pentane and the extracted culture freed from solvent by drying *in vacuo*. The material was then quickly ground with ten times its weight of freshly distilled diethylene glycol, immediately transferred to a glass-stoppered bottle and allowed to stand in the dark at room temperature for several days. Each day the suspension was gently shaken to ensure thorough mixing; vigorous shaking is to be avoided, as such treatment leads to the formation of stable foams which are difficult to handle. The suspension was finally centrifuged in the closed bowl of a Sharples supercentrifuge (25,000 r.p.m.) until it was apparently free from suspended organisms. The diethylene glycol extract was recovered as a clear pale yellow fluid. The organisms were scraped from the bowl and re-suspended in about one-half the original volume of solvent, and the whole process described above repeated. Several extracts were prepared thus. The diethylene glycol extracts were filtered through a Berkfeld candle to remove any intact organisms and cellular debris which remained. In earlier experiments the filtered solutions were dialysed at 1–2° against distilled water until free from solvent, concentrated to a small volume *in vacuo* at 15–20° and the soluble materials recovered by drying from the frozen state. The substances so obtained were sometimes found to contain a small amount of lipoid material which made them difficult to handle. In later experiments, in order to avoid this lipoid contaminant, the filtered diethylene glycol extracts were poured into 4 times their volume of cold acetone, vigorously stirred and allowed to flocculate at 1–2° for 24 hr. The materials thrown out of solution were collected by centrifugation, washed with a 1:4 diethylene glycol-acetone mixture and then with acetone. The deposits were dissolved in water, dialysed to remove acetone and the solutions evaporated to dryness from the frozen state. The amount of material recovered from 79 g. dry *T. foetus* culture by each of 5 successive diethylene glycol extracts is recorded in Table I. The quantity of substance extracted rapidly decreases, and the results indicate that for practical purposes 3 extractions remove the bulk of the material extractable with diethylene glycol.

Material precipitated from an aqueous solution of the diethylene glycol soluble material at 80 v/v per cent acetone concentration showed considerable *T. foetus* specificity, whereas that remaining in the 80 v/v per cent acetone supernatant fluid was serologically inactive. The specific substance (375 mg.) was purified by fractional precipitation from aqueous solution (16 ml.) by acetone. The solution became heavily opalescent on the addition of 11 ml. acetone and a precipitate formed on the addition of a further 1 ml. acetone. The precipitate was collected by centrifugation, washed with acetone and dried. The addition of acetone to the supernatant fluid gave additional material and in all five fractions were

collected by increasing the acetone concentration, but the general behaviour of the solution during fractionation suggested that no sharp differentiation of the materials present was to

TABLE I.—*The Extraction of Dry* T. foetus *Culture* (79 *g.*) *with Anhydrous Diethylene Glycol.*

Extraction number.	Volume of diethylene glycol added (ml.).	Volume of solvent recovered (ml.).	Amount of material in extract (mg.).	Percentage yield*.
1	800	434	435	0·55
2	600	574	275	0·31
3	450	450	140	0·18
4	300	275	40	0·05
5	200	240	34	0·04

* Expressed on the weight of dry *T. foetus* used.

be expected. The material in each fraction was dissolved in water, dialysed and recovered by drying from the frozen state. The amount of material recovered in each fraction is given in Table II. The above experiment was repeated using a further and equal amount of the

TABLE II.—*The Fractionation from Aqueous Solution by Acetone of the Material Extracted from* T. foetus.

Fraction number.	Acetone concentration (v/v per cent).	Weight of material (mg.).	End-point of specific precipitation.*
1	0–43	144	1:32,000
2	43–66	118	1:32,000
3	66–81	14	1:32,000
4	81–84	4	—
5	above 84	—	—

* Tested with the serum of rabbits immunized with living *T. foetus*.

primary diethylene glycol extract. In this instance, however, a single main fraction obtained over the range 40–80 v/v per cent acetone concentration was collected. The material recovered on the addition of acetone up to 40 v/v per cent concentration was equivalent to 5 per cent of the starting material, whereas the main fraction (40–80 v/v per cent acetone) represented rather more than 60 per cent.

A number of similar preparations of material precipitated over the range 40–80 per cent acetone concentration were mixed and the material (1·18 g.) was again fractionated from aqueous solution by the addition of acetone. Fractions separating between 0 and 43 v/v per cent, 43 and 75 v/v per cent and material remaining in solution at 75 v/v per cent acetone were obtained. Details of the experiment and analytical figures for the substances obtained are given in Table III. Fraction 2, Table III, was found to be more active serologically

TABLE III.—*Analytical Figures for the Material Obtained from* T. foetus.

Fraction number.	Acetone concentration (v/v per cent).	Amount of material recovered (mg.).	Percentage of total material recovered.	$(\alpha)_{5461}$.	N per cent.	P per cent.	End-point of specific precipitation.*
1	0–43	148	15	−60°	3·4	1·9	1:16,000
2	43–75	676	68	−62°	3·3	1·8	1:64,000
3	above 75	165	17	−56°	4·4	1·5	1:32,000

* Tested with the serum of rabbits immunized with living *T. foetus*.

than either of the other fractions, and was accordingly accepted as the best preparation of *T. foetus* specific substance and was examined in greater detail.

Properties of the Specific Substance.

Homogeneity.—The material (Table III, fraction 2) was examined in a Tiselius electrophoresis apparatus at 0° using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ. In acetate buffer pH 4, $I = 0{\cdot}1$, and in phosphate buffer pH 8, $I = 0{\cdot}2$; after prolonged electrophoresis only one component was demonstrable (Fig. 1). An ultracentrifugal examination was kindly

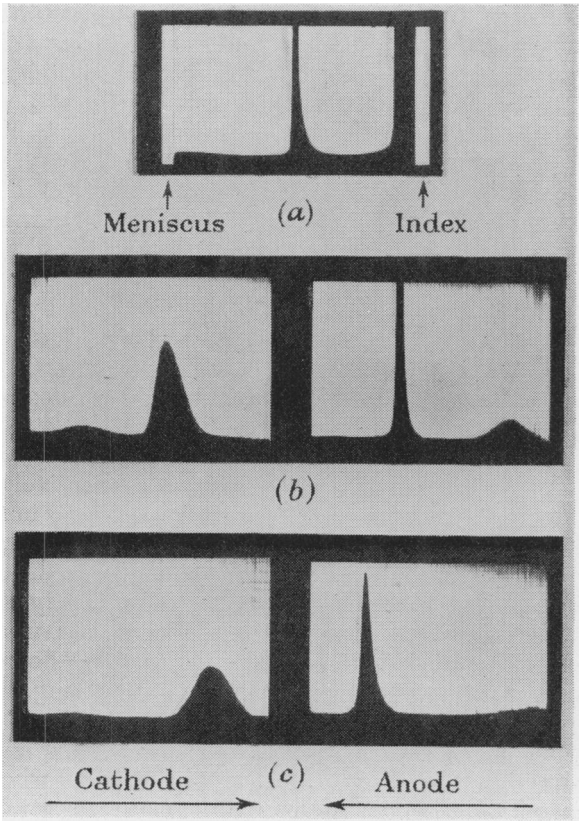

FIG. 1.—Ultracentrifuge and electrophoresis diagrams of *T. foetus* specific substance. (*a*) Ultracentrifuge diagram of a 1 w/v per cent solution in phosphate buffer (pH 8·0, $I = 0{\cdot}2$) after 50 min. at 270,000 **g**. (*b*) Electrophoresis diagram of 1·5 w/v per cent in phosphate buffer (pH 8·0, $I = 0{\cdot}2$) after 150 min. at 5 V./cm. (*c*) Electrophoresis diagram of 1·1 w/v per cent solution in acetate buffer (pH 4·0, $I = 0{\cdot}2$) after 190 min. at 5 V./cm. Migration direction in (*b*) and (*c*) shown by arrows.

carried out by Mr. H. Murray using a Svedberg oil turbine machine at 60,000 r.p.m. (270,000 g.). Solutions of the material were made up in buffers, and sufficient NaCl was present to supress any charge effect on sedimentation. The absence of spreading of the boundary after prolonged centrifugation suggests a high degree of homogeneity of particle size.

Ultraviolet absorption.—The specific substance (1 v/v per cent in water) was examined in a "Uvispek" spectrophotometer. No significant amount of absorption was shown between 260 mμ. and 310 mμ.

Optical rotation.—The specific rotation, the mean value from the examination of several preparations is $(\alpha)_{5461}$ — 62° in water (c, 1·0).

Chemical properties.—The specific substance dissolves readily in water and a 2 per cent solution gives no precipitate on the addition of an equal volume of 10 per cent trichloroacetic acid, salicylsulphonic acid (20 per cent), tannic acid (10 per cent), saturated picric acid, mercuric acetate or silver nitrate (2 per cent). The biuret and ninhydrin tests for protein and the iodine test for glycogen are negative. The material gives a strong Molisch test for carbohydrate, but does not reduce Fehling's reagent until after hydrolysis with acid. The analytical figures for a typical specimen of the specific substance are: N, 3·2 per cent; P, 1·8 per cent, none of which is free inorganic P. The methyl pentose content is 30 per cent, expressed as L-fucose. After hydrolysis with 5 N HCl for 8 hr. at 100° the values for α-amino N and α-amino-acid N reach a maximum, representing about 75 per cent and 37 per cent respectively of the total N. These determinations were kindly made by Mr. R. A. Gibbons.

The specific substance was rapidly inactivated serologically, as measured by the failure of the material to form a precipitate with a *T. foetus* immune rabbit serum, by a purified enzyme preparation obtained from a *Cl. welchii* culture filtrate (Stack and Morgan, 1949) which destroys the serological properties of the A, B and H human blood group mucoids.

Immunological properties.—Antisera containing specific agglutinins and precipitins for living *T. foetus* or its specific substance respectively were prepared by injecting living or dried cultures of *T. foetus* intravenously in rabbits.

Rabbits given 300 mg. (dry wt.) of living culture or dried organisms divided into 5 doses generally respond with the formation of specific agglutinins and precipitins, and quite potent precipitating sera are frequently obtained. These sera have been used throughout the present work to measure the serological activity of purified materials obtained from *T. foetus*. Several intradermal (0·15–0·30 mg.) and/or intravenous injections (5·0–12·0 mg.) of the purified diethylene glycol extract failed to induce any demonstrable specific agglutinin and precipitin response in a group of rabbits.

The soluble material derived from *T. foetus* (var. Manley) when tested against undiluted anti-sera to *T. foetus* (var. Manley) gave a precipitation end-point at a dilution of 1:64,000, whereas tested against an antiserum induced by immunization with *T. foetus* (var. Belfast) or against normal rabbit serum the end-titre was never more than 1:1000 and usually less than 1:200.

Kerr (1944) demonstrated that an immediate (allergic type) skin reaction can be elicited in cattle infected or immunized with *T. foetus* by a trichloroacetic acid extract of the organism ("Tricin"). It was thus of interest to determine if the purified specific hapten obtained in the present work could replace "Tricin" as a diagnostic reagent for field trials. Our results do not provide a direct answer to this question, but the following observations indicate that the development of such a reagent may be anticipated. The only cattle available to us for experimental purposes were artificially infected or immunized with *T. foetus* (var. Belfast), and were therefore unsuitable as test animals for the assay of the specific substance derived from the Manley strain. A preparation of hapten was, how-

ever, made by us from *T. foetus* (var. Belfast) and kindly tested by Dr. Kerr, who has allowed us to quote some of the results from his protocols (Table IV).

TABLE IV.—*Skin Reactions in Cows Sensitized to* T. foetus *(var. Belfast).*

Site.	Conc. test solution (mg./ml.).	Increase in skin thickness (mm.).							
		Calf E.		Heifer K 10.		Cow D 13.		Normal cow.	
		0·5 hr.	1 hr.	0·5 hr.	1 hr.	0·5 hr.	1 hr.	0·5 hr.	1 hr.
1	1·0	8·5	7·5	6·0	6·0	7·0	7·0	1·0	1·0
2	0·5	6·5	6·0	5·0	5·0	6·5	5·5	0	0
3	0·25	4·0	5·0	4·0	5·0	3·0	4·0	0	0
4	0·125	5·0	5·5	1·0	1·5	3·0	3·5	0	0

Intradermal injections (0·1 ml.) were made on the sides of the neck, the sites being numbered in clockwise direction on the left side of the neck and counter-clockwise on the right side, so that all sites with similar numbers are strictly comparable in anatomical location. The readings given in Table IV represent the increase in thickness of the double layer of skin in a fold at the site of injection, *i.e.*, the total thickness of the injected skin less the initial thickness measured with micrometer calipers before injection. The reaction is prompt, becoming evident within 10 min., reaching its peak in about 30 min.–1 hr., and thereafter fading rapidly until it has almost disappeared after 6 hr. An increase in thickness of 3 mm. or more is considered significant. The results obtained with three sensitized animals (Calf 7, Heifer K 10, and Cow D 13) and one normal cow are given in Table IV. The soluble specific substance derived from *T. foetus* (var. Belfast) produces a local swelling when injected into the skin of cattle sensitized to this organism whereas the same material injected into similar sites in a normal animal elicits no reaction. The animals were not tested with the specific soluble substance derived from the Manley variety of *T. foetus* or with other similar but unrelated polysaccharide materials ; it is therefore not possible to be certain that the reaction observed with the extract of the homologous organism is entirely specific. Further intradermal tests with the Belfast hapten (1 mg./ml.) were subsequently made by Dr. Kerr on 5 unselected bovines presented for slaughter at the Belfast abattoir. Four of the animals gave little or no reaction (0·5 to 1·0 mm.), while in the fifth, a cow, a low positive reading of 4·5 mm. was obtained.

Skin tests were also carried out on rabbits which had been given a series of intraperitoneal injections of freeze-dried *T. foetus* totalling about 800 mg. The test substances were (a) saline, (b) the glycogen-like material (see below), (c) the specific substance, and (d) laked whole *T. foetus* organisms. The intradermal injections were made on the shaved backs of the rabbits 7 days after the last intraperitoneal dose had been given, and each test material was injected as 0·1 ml. of a 0·5 w/v per cent solution. Two normal rabbits were tested similarly as controls. The results of the tests are summarized in Table V. Preparations (c) and (d) caused definite skin reactions in the rabbits (Nos. 1, 2 and 3) immunized with whole organisms, whereas materials (a) and (b) failed to induce a similar reaction. The normal animals (Nos. 6 and 7) gave no significant reaction with any of the reagents. The rabbits which reacted with reagents (c) and (d) gave a delayed type of skin reaction and in so reacting behaved very differently from

TABLE V.—*Skin Reactions Following the Intradermal Injection of Materials into Rabbits Immunized with* T. foetus *Preparations.*

Time of observation after intradermal inoculation (hr.).	Test substance.	Rabbits immunized with— Whole *T. foetus* culture. 1.	2.	3.	*T. foetus* glycogen. 4.	5.	Uninoculated rabbits. 6.	7.
0·5–1·0	a	0	±	0	±	0	±	0
	b	±	±	±	±	±	±	0
	c	+	+	+	++	+	±	±
	d	++	+	+	++	++	0	0
5	a	0	0	0	0	0	0	0
	b	0	0	0	+	0	0	0
	c	+++	++	+	+±	+	±	±
	d	+++	+++	+	+±	+	±	±
24	a	0	0	0	0	0	0	0
	b	0	0	0	0	0	0	0
	c	+++	++	++	0	±	±	±
	d	+++	++	++	0	±	±	±

a, saline. b, *T. foetus* glycogen. c, *T. foetus* specific substance. d, laked *T. foetus* culture. 0, no reaction. ±, very slight reaction. +, definite reaction. ++, strong reaction. +++, very strong reaction.

cattle, where an immediate reaction ensued. Twenty-four hours after the intradermal injection of (c) and (d) the reaction site showed a large diffuse swelling with some induration and erythema.

Acid Hydrolysis of the Specific Substance.

The substance was hydrolysed with 0·5 N and 6 N HCl in small sealed glass ampoules heated for the appropriate time in a boiling water-bath. The solutions were exactly neutralised with 0·5 N NaOH, or in the case of the stronger acid, known amounts were evaporated to dryness *in vacuo* in small glass dishes over pellets of NaOH and concentrated H_2SO_4. The rate of liberation of reducing substances and of hexosamine during hydrolysis with 0·5 N HCl is given in Fig. 2 and 3. The results indicate that the maximum liberation of reducing sugars, 46 per cent (expressed as glucose), and of hexosamine, about 9 per cent (expressed as glucosamine base), occur at approximately the same time (8–16 hr.). After heating for this time with 1 v/v per cent acetic acid the material gives about 6 per cent reduction in terms of glucose (Fig. 2), and less than 5 per cent of the total phosphorus is liberated as inorganic phosphate. The specific substance, after heating for a short time with 0·1 N HCl, gives rise to a white precipitate which dissolves readily in dilute alkali and is thrown out of solution at pH values below 7. The substance was found to contain nitrogen and organic phosphorus but has not been further investigated. The rate of liberation of phosphorus from the specific substance during hydrolysis with 2 N HCl at 100° was followed over a period of 72 hr. The results (Fig. 4) show that about 90 per cent of the total phosphorus was liberated as inorganic phosphate. Under the same conditions of hydrolysis sodium β-glycerophosphate liberated 60 per cent of its total phosphorus as inorganic phosphate. The products of complete hydrolysis with 6 N HCl for

16 hr. at 100° were examined by partition chromatography using 1 mg. of the material. The chromatogram was run in two directions at right angles with collidine (48 hr.) and phenol (20 hr.). Treatment of the chromatogram with

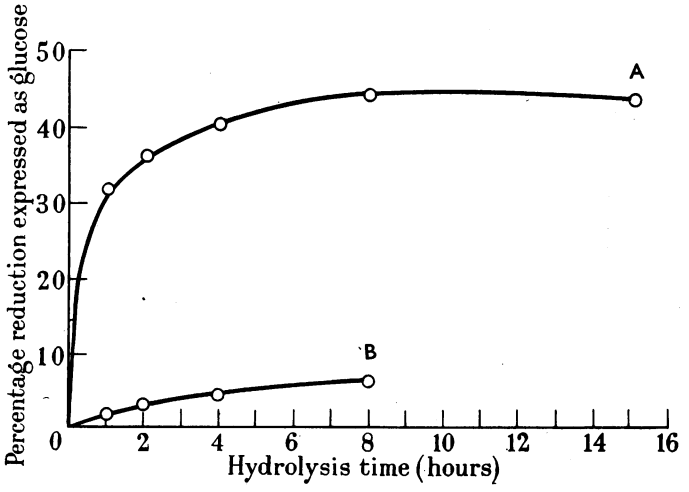

FIG. 2.—Hydrolysis of the specific substance (A) by 0·5 N HCl at 100°, (B) by N-acetic acid at 100° as determined by reducing power, expressed as glucose.

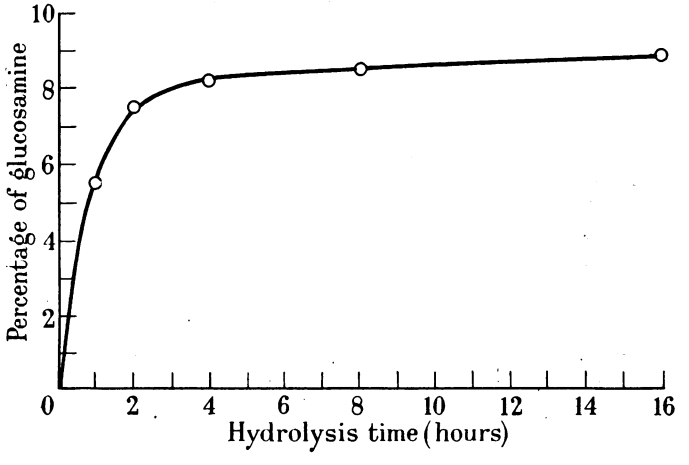

FIG. 3.—The liberation of glucosamine from the specific substance during hydrolysis with 0·5 N HCl at 100°.

ninhydrin containing 1 per cent acetic acid according to the technique of Consden and Gordon (1948) and subsequent heating at 80–90° for 5 min. developed eleven mauve spots. The chromatogram was compared with that obtained with an artificial mixture of known amino-acids run at the same time. Ten of the spots

given by the specific substance corresponded closely in position with those which were given by the following amino-acids : lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, valine and leucine (*iso*leucine).

The hexosamines, glucosamine and chondrosamine, give under these conditions greyish-mauve spots similar to those developed by amino-acids. The amino-sugars are, however, readily distinguishable from amino-acids on the chromatogram with the aid of the *p*-dimethylaminobenzaldehyde reagent described by Partridge (1948), and by the use of this reagent it was possible to identify the eleventh spot on the two-dimensional chromatogram described above as an amino-sugar. Confirmation of this conclusion was obtained by examination of the hydrolysis products of the specific substance on strip chromatograms as described earlier by Aminoff and Morgan (1948). Along the width of the paper six positions were marked out and the following substances (or mixtures) put on the paper : (*a*) chondrosamine hydrochloride, (*b*) chondrosamine hydrochloride

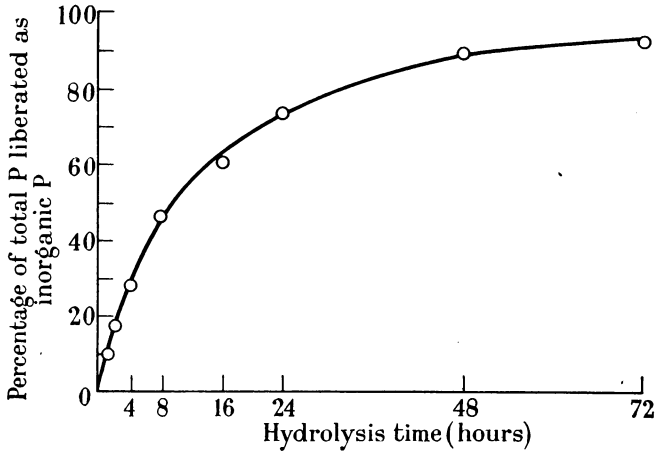

FIG. 4.—The formation of inorganic phosphate from the specific substance during hydrolysis with 2 N HCl at 100°. Results expressed as per cent of the total phosphorus liberated as inorganic phosphate.

and hydrolysis products of specific substance, (*c*) hydrolysis products alone, (*d*) chondrosamine and glucosamine hydrochlorides, (*e*) glucosamine hydrochloride and hydrolysis products of specific substance, and (*f*) glucosamine hydrochloride. The chromatogram was run with collidine for 72 hr., dried, sprayed with alkaline acetylacetone and Ehrlich's reagent and heated to 90° for 5 min. as described by Partridge (1948). A single reddish spot developed in similar areas below positions (*c*), (*e*) and (*f*), whereas two similar spots each appeared on strips (*b*) and (*d*). There is evidence, therefore, that glucosamine is the only hexosamine present in the specific substance.

One-dimensional paper chromatograms were run with collidine-water phenol-NH_3 or *n*-butanol (40 per cent), acetic acid (10 per cent) and water (50 per cent) as solvent systems, and the distribution and number and position of sugars present determined by means of $AgNO_3/NH_3$ or aniline hydrogen phthalate

reagents. Rhamnose, fucose, galactose and xylose were identified as component sugars. A weak and somewhat doubtful spot indicating the possible presence of glucose was also detected. However, owing to the presence of considerable amounts of a polyglucose in the organism, it would be unwise to accept glucose as a component of the specific substance without additional evidence.

The Isolation of a Glycogen-like Material from T. foetus.

It was observed that a material which gave an intense Molisch reaction and a typical brownish-red coloration with iodine solution was present in aqueous extracts of *T. foetus* and in thick suspensions of the organisms which had been disintegrated by ultrasonic waves. The substance possessed none of the specific serological character associated with *T. foetus*, and it seemed probable that the material was largely a storage carbohydrate similar to glycogen. In order to investigate the substance further cultures of *T. foetus* which had been exhaustively treated with anhydrous diethylene glycol and had yielded all the *T. foetus* specific substance which could be extracted under the experimental conditions with this solvent were further extracted with anhydrous ethylene glycol. By this means a carbohydrate material equivalent to between 5 and 10 per cent of the weight of the dry culture employed was selectively removed from the organism. The substance was readily recovered from the organic solvent, after it had been centrifuged at high speed and passed through a Berkfeld filter to remove any organisms or protozoal debris, by the addition of acetone to yield a concentration of 33 v/v per cent. The precipitate was re-dissolved in water and re-precipitated twice from aqueous solution with half its volume of acetone. The material gave a strongly opalescent aqueous solution which was cooled to 0°, and 4 w/v per cent trichloroacetic acid was added. The small precipitate which formed was discarded, and the main carbohydrate component recovered from solution by the addition of an equal volume of acetone. The material was dissolved in water, glacial acetic acid added to yield a concentration of 80 v/v per cent, the precipitate formed recovered by centrifugation, dissolved in water and re-precipitated at the same concentration of acetic acid. The substance, which gave a colourless opalescent aqueous solution, was thoroughly dialysed and dried from the frozen state.

The Properties of the Serologically Inactive Carbohydrate.

A 2 w/v per cent aqueous solution of the material gives an intense brownish-red coloration on the addition of iodine solution. The coloration disappears on heating the solution and reappears on cooling. A strong Molisch reaction is given by the solution, which, however, fails to reduce Fehling's solution until after treatment with hot mineral acid. The ninhydrin and biuret reactions are negative, and the addition of trichloroacetic acid (10 w/v per cent), salicylsulphonic acid (20 w/v per cent), tannic acid (10 w/v per cent), saturated aqueous picric acid, mercuric acetate and silver nitrate fail to give rise to a precipitate. The material is, however, precipitated by basic lead acetate.

The substance shows a strong dextro-rotation, $(\alpha)_{5461} + 196°$ (c, 1 per cent), and gives no pronounced or characteristic absorption bands when examined in a "Uvispek" spectrophotometer over the wave-length range 220–310 mμ. The material contains neither nitrogen nor phosphorus, and gives rise to a maximum

of about 105 per cent reduction, in terms of glucose, after hydrolysis for 8 hr. with 0·5 N HCl at 100°. The rate of hydrolysis was found to be identical with that shown by a purified preparation of liver glycogen hydrolysed under the same conditions. The hydrolysis products gave glucosazone (m.pt. 198°) when treated with phenylhydrazine. Mixed m.p. with an authentic specimen of glucosazone, 197°.

The products of hydrolysis of the carbohydrate material with 0·5 N HCl at 100° were examined on strip chromatograms using phenol or collidine as the organic phase. Glucose and acid hydrolysis products of glycogen were run on the chromatogram as control substances. The two polysaccharide materials gave identical chromatograms when the products arising from each after 30 min., 4, 16, and 24 hr. hydrolysis were compared. After complete hydrolysis of the serologically inactive carbohydrate, glucose was the only sugar demonstrable on the chromatogram. The carbohydrate material derived from *T. foetus* appears to be a polyglucose, indistinguishable from glycogen by the tests carried out.

Immunological Observations.

In view of the observations of Campbell (1936, 1937, 1939, 1942) on the antigenic nature of a series of nitrogen-free polysaccharides which he obtained from certain metazoon organisms, such as *Ascaris lumbricoides*, *Trichinella spiralis* and *Cysticercus crassicollis*, it seemed expedient to examine the immunological behaviour of the glycogen obtained from *T. foetus*. Two rabbits (Table V, No. 4 and 5) were inoculated intraperitoneally at 2–3-day intervals with 15 mg. of the material. Two series of inoculations were given, each of 5 doses and separated by an interval of 5 days. In the last series 20 mg. of the material was given at each injection. Three rabbits (Table V, No. 1, 2 and 3) were similarly immunized each with 800 mg. of freeze-dried *T. foetus* culture. The individual doses varied from 100 mg. to 300 mg. of the dry culture. Two normal rabbits served as controls. Seven days after the last dose each animal was bled and skin-tested. The two rabbits which had received the glycogen-like material and the two normal rabbits showed no significant skin reaction when given the glycogen-like material intradermally (0·1 ml. of 0·5 w/v per cent solution in saline). The two animals which had been immunized with the glycogen-like material also received intradermally the freeze-dried *T. foetus* culture and the specific substance, and gave a definite and immediate reaction which lasted for at least 5 hr. and then disappeared completely. The three rabbits which had been immunized with the whole culture of *T. foetus* gave no skin reaction on receiving the glycogen-like material intradermally. The results of these tests are given in Table V.

Precipitation reaction.—In no instance did the sera of the rabbits which had received intraperitoneal doses of either the whole culture of *T. foetus* or the purified protozoal glycogen give a precipitate on addition of the homologous glycogen preparation.

DISCUSSION.

A method for the isolation of an immunologically specific substance and a glycogen-like carbohydrate from the protozoon, *T. foetus*, is given which is based on the differential extraction of the two materials by means of anhydrous organic

solvents. The possibility of elaborating such a method for the isolation of individual components from the bacterial cell was discussed earlier (Morgan, 1937; Morgan and Partridge, 1941), and such a procedure involving the successive extraction of a thoroughly washed and freeze-dried culture of *T. foetus* with diethylene glycol and ethylene glycol is now described. Anhydrous diethylene glycol removes, at normal temperatures and a neutral pH, not more than a few per cent of the protozoal substance, and the extract contains a substance which possesses a serological specificity very similar to, or identical with, that of the dominant specificity of the living organism. This material, after fractionation from aqueous solution with acetone to remove small amounts of unspecific contaminants, is identified as a polysaccharide-amino-acid complex which precipitates at low concentrations with a *T. foetus* immune rabbit serum but fails to engender specific agglutinins or precipitins in rabbits under the conditions of immunization employed. These results suggest that the specific substance obtained is probably not the complete antigen as it exists in the living or freeze-dried culture, but that it is an important constituent of the antigen and functions as the specific determinant, and might be classified provisionally as a hapten. The absence of antigenicity when the material is tested in rabbits is similar to that recorded for preparations of the human blood-group substances, which are likewise complexes of polysaccharide and amino-acid-containing residues. The blood-group substances are, however, powerful antigens when tested in man (see Witebsky, Klendshoj and McNeil, 1944; Wiener, Soble and Polivka, 1945), and it may well be that in a different animal species the specific material obtained from *T. foetus* would similarly prove capable of inducing the formation of specific antibodies.

Although only preliminary observations on the chemistry of the specific substance have yet been made, it is already evident that this immunologically important material consists of a complex carbohydrate to which are firmly bound amino-acids, most probably in the form of a complex amino-acid-containing residue. Quantitatively, the main sugar component (30 per cent) is methylpentose, which is, however, not composed of a single sugar but is a mixture of fucose and rhamnose. Chromatographic analysis reveals the presence of galactose and xylose and the amino-sugar glucosamine as well as the two methylpentoses. The low content of glucosamine (9 per cent) in the specific substance suggests that most of the nitrogen (3·2 per cent) in the material arises from the amino-acids present, which are, incidentally, found to be similar qualitatively to those present in the human specific blood-group substances. Aromatic and sulphur-containing amino-acids appear to be absent. The organic phosphorus is converted slowly to inorganic phosphate during hydrolysis of the specific substance with 2 N HCl at 100°, but there still remains after 72 hours about 10 per cent of the total phosphorus in organic combination. There is no evidence that the hexosamine liberated during hydrolysis fails to reach its maximum value before the whole of the phosphorus is liberated as inorganic phosphorus, as has been reported by Bendich and Chargaff (1946) for the O antigen of Proteus OX 19.

Compared with many bacteria *T. foetus* in the living or in the dried state is a poor antigen. However, antibodies specific for *T. foetus* are readily produced by injecting whole cultures or freeze-dried preparations into rabbits. The specific soluble substance isolated from *T. foetus* (var. Manley), the strain commonly met with in England, when tested with an antiserum to *T. foetus* (var.

Belfast), a strain predominant in Northern Ireland, shows only a weak cross-reactivity, and indicates that the specific substance of the variety Manley is serologically distinct from that of the Belfast variant.

The problems of immunity, sensitization and desensitization in bovines infected or immunized with *T. foetus* have been studied in considerable detail in recent years (Kerr and Robertson, 1941, 1943, 1945, 1946, 1947; Kerr, 1944; Kerr, McGirr and Robertson, 1949), and it has been established in these studies that the value of the skin test in carefully controlled animal experiments is very considerable. Until recently a skin-testing reagent was made from *T. foetus* cultures by laking them with water and precipitating a material from the supernatant fluid with trichloroacetic acid, and the use of the specific material prepared by the extraction of *T. foetus* with ethylene glycol for skin testing has already been favourably commented on by Kerr, McGirr and Robertson (1949), Kerr, Robertson and McGirr (1951), who state that the substance causes no desensitization even when the tests are repeated daily. The results of the skin-testing experiments described in the present paper, using the purified diethylene glycol extract of *T. foetus*, although in many respects inadequate, support these authors' conclusion that the purified specific substance is a useful reagent for the detection of the sensitized state in immunized animals. The absolute specificity of the preparation of the specific substance is not yet completely established, but it is to be hoped that further field experiments may be undertaken.

After repeated extraction of the freeze-dried *T. foetus* culture with anhydrous diethylene glycol no further material passes into solution. If the solvent is now changed and anhydrous ethylene glycol is used a different cellular component, which amounts finally to rather less than 10 per cent of the dry weight of the *T. foetus* culture used, is readily extracted. After several extractions ethylene glycol also fails to remove further material, and to obtain additional constituents from the protozoal bodies it is necessary to employ yet another solvent system. Ethylene glycol, however, is less specific as an extracting agent than is diethylene glycol, and some nucleic acid and nucleoprotein is obtained along with the glycogen-like polysaccharide. Preliminary studies have shown that the nucleoprotein substance possesses *T. foetus* specificity, and it is probably this material which is responsible for reactivity of the " Tricin " and ethylene glycol extracts used by Kerr, McGirr and Robertson (1949, 1951). These materials are separated by repeated precipitation of the polysaccharide from aqueous solution by 33 v/v per cent acetone.

The polysaccharide material in the ethylene glycol extract, after purification, is found to be free from nitrogen, strongly dextro-rotary, and yields glucose only after hydrolysis with mineral acid. This polysaccharide, which possesses no *T. foetus* specific serological character, is most probably a reserve carbohydrate, and is similar to, if not identical with, glycogen. The material fails to induce in the rabbit the formation of anti-sera which precipitate specifically with glycogen, and therefore it behaves in this respect as does mammalian liver glycogen, and is not similar in behaviour to the glycogen isolated from the nematode, *Ascaris lumbricoides*, which according to Campbell (1936) can produce precipitins specific for glycogen in the rabbit.

The *T. foetus* cells after exhaustive extraction with diethylene glycol and ethylene glycol still induce in the rabbit the formation of precipitins and agglutinins specific for *T. foetus*. This observation suggests that the treated cells still

retain a part of the specific substance associated with a cellular component which endows it with full antigenic properties.

SUMMARY.

A method is described for the isolation and purification of a serologically specific substance from the protozoon, *Trichomonas foetus* (var. Manley).

The specific substance, which contains 3·2 per cent N and 1·8 per cent P, is composed of a polysaccharide and an amino-acid-containing moiety, and is strongly laevo-rotatory, $(\alpha)_{5461} - 62°$. It fails to induce the formation of specific agglutinins or precipitins in the rabbit and shows only slight cross-reactivity with *T. foetus* (var. Belfast).

A preliminary examination of the polysaccharide component shows it to contain rhamnose, fucose, xylose, galactose and glucosamine. The amino-acid-containing moiety gives rise to ten amino-acids on hydrolysis.

A polyglucose, most probably glycogen, is identified as a cellular component of *T. foetus*.

The immunological behaviour of the materials isolated is discussed.

The authors wish to express their thanks to Dr. Muriel Robertson, F.R.S. for much helpful advice on the growth of pure cultures of *T. foetus*, and to Dr. D. W. Henderson of the Microbiological Research Department, Porton, for a large quantity of *T. foetus* culture. One of the authors (J. G. F.) is indebted to the United States Veterans Administration for an Educational Grant. We wish to acknowledge the technical assistance of Mr. H. Murray of the Department of Biophysics of the Lister Institute. The investigation was aided by a grant from the Nuffield Trust.

REFERENCES.

AMINOFF, D., AND MORGAN, W. T. J.—(1948) *Nature, Lond.*, **162**, 579.
BENDICH, A., AND CHARGAFF, E.—(1946) *J. biol. Chem.*, **166**, 283.
CAMPBELL, D. H.—(1936) *J. infect. Dis.*, **59**, 266.—(1937) *Proc. Soc. exp. Biol., N.Y.*, **36**, 511.—(1939) *J. infect. Dis.*, **65**, 12.—(1942) *Science*, **96**, 431.
CONSDEN, R., AND GORDON, A. H.—(1948) *Nature, Lond.*, **164**, 443.
Idem AND MARTIN, A. J. P.—(1944) *Biochem. J.*, **38**, 224.
DISCHE, Z., and SHETTLES, L. B.—(1948) *J. biol. Chem.*, **175**, 595.
DUBOS, R. J.—(1948) ' Bacterial and Mycotic Infections of Man.' Philadelphia (Lippincott).
ELSON, L. A., AND MORGAN, W. T. J.—(1933) *Biochem. J.*, **29**, 1824.
FEINBERG, J. G.—(1952) *J. Path. Bact.*, **64**, 645.
FULLER, A.—(1938) *Brit. J. exp. Path.*, **19**, 130.
HENDERSON, D. W., AND MORGAN, W. T. J.—(1938) *Ibid.*, **19**, 82.
KERR, W. R.—(1944) *Vet. Rec.*, **56**, 303.
Idem AND ROBERTSON, M.—(1941) *Vet. J.*, **97**, 351.—(1943) *J. comp. Path.*, **53**, 280.—(1945) *Vet. Rec.*, **57**, 221.—(1946) *J. comp. Path.*, **56**, 38, 101.—(1947) *Ibid.*, **57**, 301.
Idem, MCGIRR, J. L., AND ROBERTSON, M.—(1949) *Ibid.*, **59**, 133.
Idem, ROBERTSON, M., AND MCGIRR, J. L.—(1951) *J. Hyg., Camb.*, **49**, 67.
MA, T. S., AND ZUAZAGA, G.—(1942) *Industr. Engng. Chem. (Anal.)*, **14**, 280.
MACDONALD, E. M., AND TATUM, A. L.—(1948) *J. Immunol.*, **59**, 309.

MARKHAM, R.—(1942) *Biochem. J.*, **36**, 790.
MARTLAND, M., AND ROBISON, R.—(1926) *Ibid.*, **20**, 847.
MORGAN, B. B.—(1948) *J. cell. comp. Physiol.*, **32**, 235.
MORGAN, W. T. J.—(1937) *Biochem. J.*, **31**, 2003.—(1949) 'The Nature of the Bacterial Surface.' Oxford (Blackwell).
Idem AND PARTRIDGE, S. M.—(1940) *Biochem. J.*, **34**, 169.—(1941) *Ibid.*, **35**, 1140.
PARTRIDGE, S. M.—(1946) *Nature, Lond.*, **158**, 270.—(1948) *Biochem. J.*, **42**, 238.—(1949) *Nature, Lond.*, **164**, 443.
Idem AND MORGAN, W. T. J.—(1942) *Brit. J. exp. Path.*, **23**, 84.
ROBERTSON, M.—(1941) *J. Path. Bact.*, **53**, 391.
STACK, M. V., AND MORGAN, W. T. J.—(1949) *Brit. J. exp. Path.*, **30**, 470.
SVEC, M.—(1944) *J. Bact.*, **47**, 505.
SOMOGYI, M.—(1937) *J. biol. Chem.*, **117**, 771.
WIENER, A. S., SOBLE, R., AND POLIVKA, H.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **58**, 310.
WITEBSKY, E., KLENDSHOJ, N. C., AND MCNEIL, C.—(1944) *Ibid.*, **55**, 167.