THE PREPARATION AND PROPERTIES OF ENZYMES FROM *CL. WELCHII* (TYPE B) FILTRATES WHICH DESTROY BLOOD-GROUP SUBSTANCES.

M. V. STACK AND W. T. J. MORGAN.

From the Lister Institute of Preventive Medicine, Elstree and London.

Received for publication August 12, 1949.

SCHIFF (1935, 1939) first observed that cultures and culture filtrates from certain strains of *Clostridium welchii* possess the power to destroy the specific serological properties of the blood-group A-substance present in commercial peptone and human saliva. Schiff considered the enzyme to be specific for the A-substance and to be without action on the Group B factor in saliva.

Schiff and Weiler (1931*a*) observed that the normal faeces of persons of all groups and of certain animals contain an enzyme which readily inactivates the A and B blood-group substances. The enzyme is also present in saliva (Schiff and Weiler, 1931*b*), and is found in the saliva of secretors and non-secretors alike (Matson and Brady, 1936). The enzyme does not appear to be derived from the bacteria found in the mouth, and it has been suggested by Schiff and Burón (1935) that the enzyme is secreted by the glandular cells. Landsteiner and Chase (1935) reported that A-substance was decomposed by an organism, *Pullulomyxa botrytis* (Thaysen, 1939), which was isolated by Morgan and Thaysen (1933), and shown to be active in decomposing a number of specific bacterial polysaccharides.

The isolation of the specific blood-group substances of animal and human origin in a purified form led to a reinvestigation of the enzymic destruction of their serological properties (Morgan, 1946). A number of *Cl. welchii* (Type A) culture filtrates, crude and partially purified with reference to collagenase (κ-toxin), an enzyme which breaks down collagen (Oakley, Warrack and van Heyningen, 1946 ; Bidwell and van Heyningen, 1948 ; Bidwell, 1949), were examined, and were found to contain enzymes which would rapidly inactivate the purified A, B and H blood-group substances. Increase in activity of the collagenase, hyaluronidase and certain toxins common to these filtrates during their purification and concentration, however, was not correlated with increased capacity to inactivate the group mucoids, and it appeared that they were not the factors responsible for the decomposition of the blood-group substances. Nor does the λ-enzyme of Oakley, Warrack and Warren (1948) appear to be involved, since after purification of a *Cl. welchii* (Type B) culture filtrate with a resultant 50 per cent yield of this enzyme, only 2 per cent of the A-enzyme remained. Heating the culture filtrates for 1 hour at 56° resulted in the inactivation of the enzymes which destroy the A- and B-substances, but left unimpaired the enzyme which decomposed the so-called O-substance, now referred to as H-substance (Morgan and Watkins, 1948). The results of these investigations indicate that at least two enzymic activities are displayed by the partially purified culture filtrates.

The activity which destroys the serological properties of the A and B group substances is thermolabile; the other enzymic activity is relatively thermostable and destroys only the H-substance.

The examination of culture filtrates from selected strains of *Cl. welchii* (Type B) has shown that enzyme preparations of much greater activity can be obtained readily, and the work described in this communication concerns the purification and properties of the group specific enzyme preparations obtained from Type B strains. The intended separation of the enzymic activities has not been achieved, and therefore the A- or B-enzymes cannot yet be obtained free from H-enzymic activity. A preliminary account of this work has already appeared (Stack and Morgan, 1948).

EXPERIMENTAL.

Materials and Methods.

Cultures.

The *Cl. welchii* (Type B) strain used by Schiff (1939), also strains CN 1253 and CN 1990, were found to yield the most active filtrates encountered. Fourteen other strains examined after two subcultures on peptone medium were found to yield not more than one-fifth of the enzyme titre found in the culture filtrates of the three chosen strains. Another strain, originally producing less enzyme than these, produced filtrates of maximum activity after three subcultures had been made. Culture filtrates from representative Type A, C and D strains contained about one-tenth of the enzyme activity found for average Type B strains. Tests on culture filtrates from a few strains of *Cl. histolyticum*, *B. anthracis*, *Staph. aureus*, *Ps. pyocyanea* and *V. cholerae* failed to reveal the presence of enzymes able to inactivate the blood-group substances.

The organisms were grown on a peptone medium consisting of Evans peptone, 3 per cent, and sodium β-glycerophosphate, 2·5 per cent, supplemented before inoculation with glucose, 2 ml. per 100 ml. of a 10 per cent solution, and thiolacetic acid, 2 ml. per 100 ml. of a 0·4 per cent. solution. Without glucose, enzyme yields were generally one-third less, but without thiolacetate, only one quarter of the enzyme production occurred. Glucose caused no stimulation of enzyme production unless thiolacetate was present. The addition of 0·01–0·20 per cent of the test substrate caused no adaptive response. As group substances are present in the peptone used in the medium, the lack of adaptive response was confirmed by growing the same strain in the simplified medium described by Rogers (1945) with or without the addition of purified pig gastric mucin (A- and H-substances).

Specific blood-group substrates.

The test-substrate employed, unless otherwise stated, was a purified mucoid material obtained from a commercial preparation of pig gastric mucin according to the method described by Morgan and King (1943). The substance possessed A and H, the so-called O, activity. Recent work has shown that the O specificity of the mucoid is not the same as would be expected if the material were a product of the *O* gene. For this reason Morgan and Watkins (1948) suggested that the so-called O character would be better designated by the letter H, as this symbol would serve to emphasize that it is not a product of the *O* gene, but that it is a

heterogenetic material common to the great majority of human erythrocytes, irrespective of their A B O Group.

In a few experiments a preparation of human A-substance was employed which showed no H-activity, and on the basis of its chemical, physical and immunological properties was most probably homogeneous. Similarly, carefully purified H-substance obtained from pseudomucinous ovarian cyst fluids was used in other experiments. A potent specimen of ovarian cyst fluid obtained from a Group B secretor served as a source of B-substance. These materials were readily inactivated by the enzyme preparations.

For convenience in recording the results the enzymic activities which destroy the A-, B- and H-substrates are assumed to be due to separate enzymes and are termed A-enzyme, B-enzyme and H-enzyme respectively. The properties of the B-enzyme have not been examined in detail, but in those examined they do not appear to be distinguishable from those of the A-enzyme, except in their group specificity.

Measurement of enzymic activity.

The amount of the specific substrate, present at any given time after mixing with the enzyme preparation, was determined by means of the agglutination inhibition test described earlier (Morgan and van Heyningen, 1944). The anti-A, anti-B and anti-H sera used throughout the investigation were frequently titrated in inhibition tests against " standard " preparations of A-, B- and H-substances, respectively. The sera were stored in 2 to 3 ml. amounts without antiseptic and in sealed ampoules at − 10°.

Ideally, the amount of change taking place over a short interval of time and in the presence of a large excess of unchanged substrate should be measured to determine the activity of an enzyme preparation. In this instance, however, measurements of this kind cannot be made owing to the insensitive nature of the agglutination inhibition test, which measures the excess antibody left in the system after the undecomposed group substance has neutralized a part of the standard dose of agglutinin. Agglutinin and, therefore, substrate concentrations differing by 50 per cent can readily be determined, and differences in concentration of this order are usually employed in agglutinin titration tests. If care is taken and all dilutions are made with graduated pipettes in place of the Pasteur pipettes usually employed in this type of titration, differences of 33 per cent in agglutinin concentration can be detected. For the simple purpose of following the change in activity of an enzyme preparation, for example during purification, 50 per cent differences, as derived by making progressively double dilutions of the enzyme-substrate system, are convenient, and have been used in this investigation.

A unit of enzymic activity is defined as that amount of activity which will bring about an extent of inactivation of 1·0 ml. of 0·10 per cent substrate in 2 hours at 37° and pH 7·0, which results in the lowering of the inhibition titre by four tubes when the usual two-fold geometrical dilution scale is employed. This unit is equivalent to the destruction of 94 per cent of the serological activity of 1·0 ml. of a 0·10 per cent solution of the blood-group substance.

Tests for the measurement of enzymic activity were carried out as follows: The group substance, 1·0 ml. of a 0·10 per cent solution, was mixed with different amounts of the enzyme preparation contained in 1·0 ml. of McIlvaine buffer, pH 6·8–7·0. The mixture was incubated at 37° for 2 hours in the presence of toluene

and then heated for 10 min. at 100°. The latter step is essential, otherwise the enzyme preparation brings about a sensitization and false agglutination of the red cells used in the agglutination-inhibition test subsequently employed to measure the extent of inactivation of the substrate.

The results of a typical titration carried out to determine the activity of an enzyme preparation are set out in Table I, from which it will be seen that 0·80 mg. of the enzymic material brought about a lowering of the inhibition end-point by four tubes, i.e. decomposed 94 per cent of the substrate under the standard test conditions. By definition, this amount, 0·80 mg., of the preparation contains one unit of activity. With the same laboratory " standard " a parallel series of results was obtained for the H-enzyme tests.

TABLE I.—*Showing the Inactivation of A-substance by Enzyme in 2 Hours at 37°, pH 7·0.*

Enzyme (mg. per ml.).	Inhibition of agglutination of A-cells by anti-A serum after the addition of the following dilutions of 0·10 per cent substrate:									Residual substrate (per cent).	Calculated A-enzyme (units/mg.).
	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256	1 : 512.		
0	0	0	0	0	0	0	0	1	2	100	—
0·2	0	0	0	0	0	0	1	2	3	50	(1·2)
0·4	0	0	0	0	0	1	2	3	4	25	1·2
0·6	0	0	0	0	1	2	3	4	4	12	1·2
0·8	0	0	0	*1*	2	3	4	4	4	*6*	*1·2*
1·0	0	0	1	2	2	3	4	4	4	3	1·2
1·2	0	1	2	3	4	4	4	4	4	1·6	1·2
1·6	1	2	3	3	4	4	4	4	4	0·8	1·1

0 = Absence of agglutination; 1 = groups of a few cells (the end-point); 2 = larger groups, many free cells; 3 = clumps visible without magnification; 4 = macroscopic agglutination.

In view of the insensitive nature of the agglutination inhibition test it is advisable to determine as closely as possible the amount of enzyme which will bring about a lowering by four tubes of the inhibition end-point of the standard substrate, measured under the defined conditions. When many determinations of activity are involved, however, as is inevitable for example in following the efficiency of purification procedures, greater or less inactivation than 94 per cent is frequently recorded, and an empirical interpretation of the end-point titres given in Table I has been used for the determination of the activity of the preparations in terms of the proposed units.

It has been found that if the difference in the number (N) of two-fold dilutions required to give the agglutination inhibition end-point before and after the enzyme has acted, be multiplied by the initial quantity of substrate (1·0 mg.) and divided by the amount of enzyme used, the result is approximately constant. The above definition of the unit employed can therefore be expressed in the working formula:

$$\text{Enzyme units } \frac{N}{4} \times \frac{\text{Initial substrate}}{\text{Initial enzyme}}$$

Table I contains the titration results obtained in one particular experiment in which the enzyme-substrate ratio was varied 5-fold. The calculated activity in units is in each instance the same within the experimental error. Similar

results were obtained when the H-enzyme and its substrate were examined. The extent of substrate destruction by an appropriate amount of enzyme was not affected by ten-fold dilution of the system before incubation or by employing an acetate buffer.

Enzyme Production during Growth of Cl. welchii *(Type B).*

Culture filtrates show maximum activity as soon as vigorous gas evolution ceases, usually about 12 to 15 hours after inoculation. The A-enzyme reaches its maximum titre at this time and then declines, whereas the H-enzyme, which is more stable under these conditions, remains at this optimal level for at least another day. The results of numerous experiments showed that the production of the enzyme occurs equally well whether the medium fills small or large vessels. The best yields are obtained when a 1 per cent inoculum from a vigorously growing subculture is added to the warm medium, freshly supplemented with glucose and thiolacetic acid. Poorer yields of A-enzyme, which, however, showed greater specific activity, could be obtained by growing the organisms in peptone medium contained in a cellophane dialysis sac, surrounded by fresh medium at 37°.

Stability of Enzyme Solutions.

Cultures were centrifuged and the clear supernatant fluids stored at 0 to 2° under toluene. If used within a day or so, the supernatant fluids show good enzymic activity against A-, B- and H-substances. If required for use more than a week later storage at − 10° without preservative is essential, but not always successful for the A-enzyme. The H-enzyme is fairly stable in aqueous solution at 0°. Drying a fresh culture filtrate from the frozen state prevents rapid deterioration of the enzymes. Precipitation of the enzymes by the addition of solid ammonium sulphate to the culture medium gives rise to a sticky scum, which partially dissolves in 80 per cent of glycerol to yield a stable enzyme solution. The addition of 0·4 per cent of gelatin, gum arabic, or peptone, does not stabilize aqueous solutions of the A- and B-enzymes. The stability of the enzymes was examined at pH levels between 3 and 12 by incubating for an hour at 20° before readjusting to pH 7·0 and testing. It was found that the A- and H-enzymes retained their full activity under these conditions from pH 5 up to a value as high as pH 11.

In confirmation of the earlier results of Morgan (1946), it was found that incubation in the absence of substrate for one hour at 56° destroys the A- and B-enzymes and such heated preparations can be used for control purposes. Fig. 1 shows that the A- and H-enzymes can be qualitatively differentiated in the one direction by heating at 55° for 10 minutes owing to the relative stability of the H-enzyme in buffer (pH 7·0) at this temperature. A preliminary incubation of the enzyme-substrate mixture at 55° or 60° for 10 minutes prior to the usual 2-hour reaction period at 37° reveals that the A-enzyme is protected at 55° by its substrate, but not at 60°, while the H-enzyme is protected at 60°.

If, after the usual enzyme-substrate incubation for 2 hours at 37°, a further 1·0 mg. amount of substrate be added, it has been found that on repeating the incubation, complete inactivation of the additional H-substance by the H-enzyme occurs. However, the A-enzyme is unable to inactivate the extra A-substrate completely—a finding which is not unexpected in view of the more labile character

of the A-enzyme, which presumably loses the protection of its substrate towards the end of the first incubation period.

Unit quantities of the purified enzymes in phosphate buffer were incubated for 2 hours at 37° with equal volumes of 0·02 M solutions of the following substances: Potassium cyanide, formaldehyde, glycerol, ascorbic acid, phenol, manganous chloride, ferric chloride, lead acetate, and silver nitrate. After further incubation with substrate, the H-enzyme titre was found to be enhanced by the first and depressed by the last three substances. Inhibition only occurred in tests where precipitation of the metal phosphates occurred. The serological test was not affected in blank experiments by the substances which appeared to interfere with the enzyme. These results are similar to those reported by Schiff and Weiler (1931*b*) and by Stimpfl (1932).

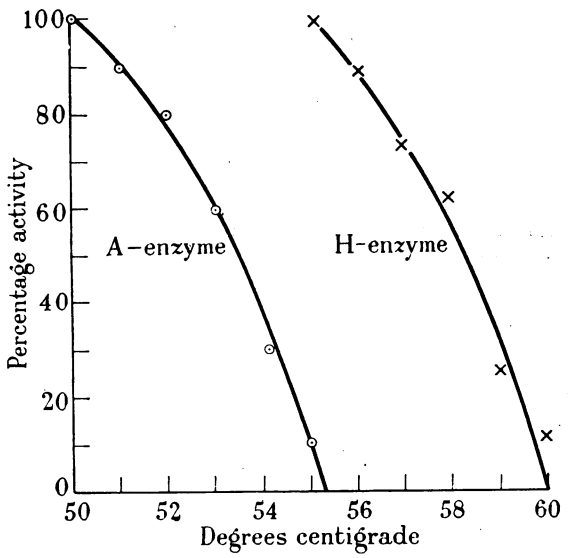

FIG. 1.—Differential inactivation of A- and H- enzymes by incubation for 10 minutes over the range 50–60°.

Optimum Temperature and pH of Reaction.

The optimum temperature for the reaction between the A- and H-enzymes and their substrates was investigated by incubation at pH 7 for 2 hours. The results indicated that both enzymes are most active over the range 40–45°, and still appreciably inactivate their substrates at temperatures above 50°. Fig. 2 is a composite curve based on over fifty readings averaged over 5° temperature intervals in order to detect the small differences in activity observed between 30° and 50°, measurements on both A- and H-enzymes being combined, since there were no observable differences between them. This conclusion is not at variance with the evidence on temperature stability, for in the latter experiment no substrate was present.

The rate of enzyme action at 37° was approximately doubled at 47° and halved at 27°. The results set out in Fig. 3 show that purified enzymes can be assayed in 10 minutes instead of the 2 hours required for culture filtrates. Inactivation

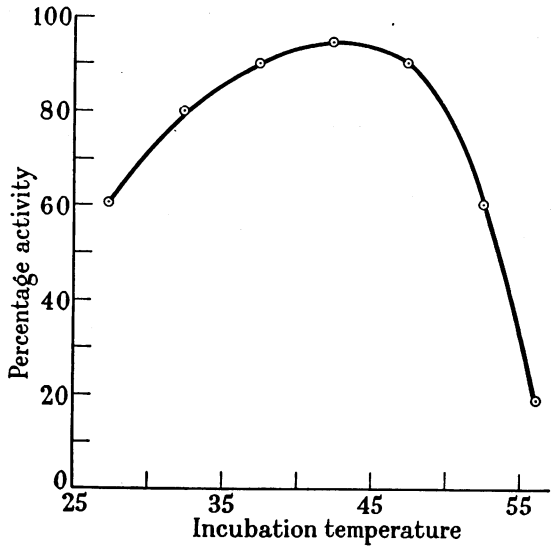

FIG. 2.—The variation of enzyme activity with temperature. Showing the mean values over 5° ranges. Extreme values differ by ± 20 per cent from those plotted.

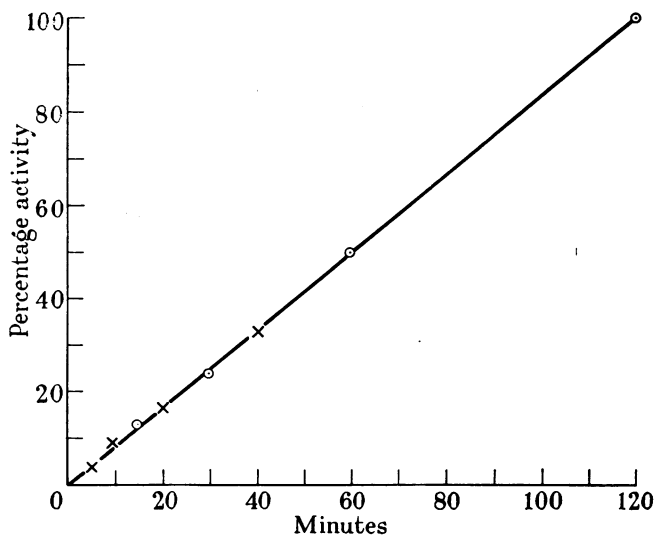

FIG. 3.—Showing the relationship between incubation period and enzyme activity calculated from (*a*) the enzyme-substrate ratio, which was progressively halved as the incubation period was doubled, and (*b*) the constant inactivation of the substrate resulting from this procedure.

of the substrate to the same extent was obtained by keeping constant the product of enzyme concentration and incubation period.

To determine the influence of pH on the activity of the enzymes a standard enzyme preparation was mixed with 0·10 per cent substrate in the presence of citrate, phosphate and borate buffers to give pH values between 3·7 and 9·8. The mixtures were incubated at 37° for 2 hours, readjusted to pH 7·0 and heated at 100° for 10 minutes before titration for undecomposed blood-group substance. The averaged results of a number of experiments compose the curves given in Fig. 4, from which it will be seen that there is a steady increase in A-enzyme activity from pH 3·7 to about pH 5·5. The activity then falls until at pH values

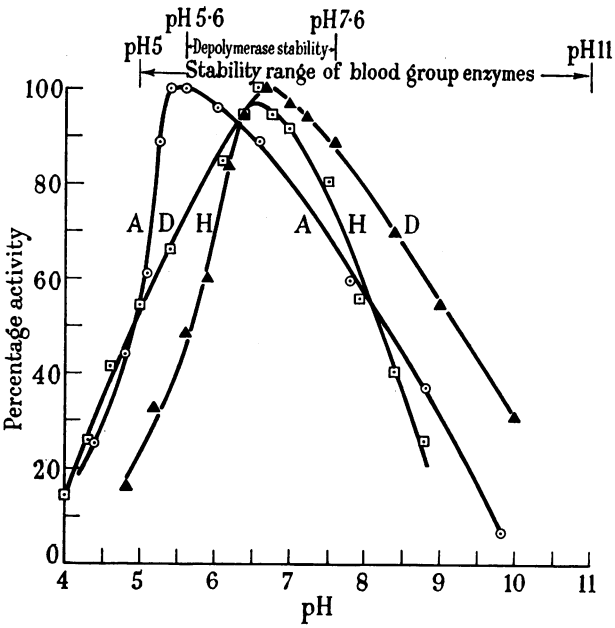

FIG. 4.—The variation of enzyme activity between pH 4 and10. A (⊙——⊙) A-enzyme : H (▲——▲) H-enzyme ; D (⊡——⊡) depolymerase.

above 9 no significant inactivation of the specific substrate occurs. The H-enzyme behaves similarly, but shows its optimum activity at about pH 6·5. In both instances, however, over 90 per cent of the maximum activity is shown over a range of 1·2 pH units. In following the isolation and purification of the enzymes which will now be described, the measurement of enzymic activity has been carried out within the pH range 6·8–7·0, which provided a favourable condition for enzyme action, destruction of residual enzyme, and titration of residual substrate.

The Purification of the Blood-Group Enzymes.

The assessment of the specific activity of materials obtained as a result of fractionation of crude culture supernatant fluids was made by expressing the activity in terms of mg. " tyrosine " or mg. of nitrogen. " Tyrosine " was deter-

mined by means of a photo-electric colorimeter after the addition of sodium carbonate and Folin and Ciocalteu's reagent (1927) to samples containing 20–40 μg. " tyrosine " (light filter : Ilford 204). Nitrogen was similarly determined with Nessler's reagent (light filter : Ilford 302) after samples of the enzyme preparations, containing 20–40 μg. N, had been digested with H_2SO_4 and H_2O_2, using a procedure similar to that described by Miller and Miller (1948). The more convenient estimation of " tyrosine " was permissible, since the quantity of nitrogen present in purified material was consistently 3·7–3·8 times greater than the " tyrosine " content.

The addition of 70 g. per cent $(NH_4)_2SO_4$ to fresh culture supernatant fluids gives rise to protein scums which are partly soluble in aqueous buffer at pH 7. By this means the specific activity is increased about eight-fold for a 30 per cent recovery of the enzymes.

Further similar experiments demonstrate that the most active material, 50 H-enzyme units per mg. tyrosine, is thrown out of solution between 40 and 43 per cent added $(NH_4)_2SO_4$, but this fraction accounts for only a small part of the total activity.

Attempts to remove impurities from crude enzyme preparations by simple dialysis were without success owing to the extensive loss of enzymic activity. Ultrafiltration by means of dialysis sacs, strengthened by silk jackets and kept under reduced external pressure, reduced the fluid to one-tenth of its original volume. A considerable degree of purification is achieved by this means, but again the total activity recovered indicates that an extensive loss occurs.

Shaking culture filtrates or partially purified material in McIlvaine buffer at pH 7 with alumina, bentonite, calcium phosphate, Celite, Decalso, Fuller's earth, kaolin and magnesium silicate failed to bring about a separation of the enzymes or to remove useful amounts of impurities. Passage of the mixed enzymes through columns of charcoal-glass powder mixtures usually resulted in the adsorption of at least 50 per cent of the A-enzyme activity, together with 30 per cent or so of the total solids in the preparation. Passage through columns of alumina or calcium phosphate, however, allowed half the H-enzyme to be recovered in the filtrate with very little A-enzyme. On no occasion was there evidence that the adsorbed enzymes could be eluted by altering the pH or by running moderate concentrations of electrolytes through the columns.

Attempts to adsorb the enzymes on charcoal in the presence of 3M $(NH_4)_2SO_4$ were more successful, for subsequent elution from the charcoal with 1M $(NH_4)_2SO_4$ or buffer at pH 7·0 gave satisfactory yields of enzymes of considerably enhanced purity. For example, 1·0 g. of dried crude culture filtrate was dissolved in 20 ml. 3M $(NH_4)_2SO_4$ and shaken gently with 0·40 g. charcoal. The charcoal was recovered and eluted successively with 2M $(NH_4)_2SO_4$, 1M $(NH_4)_2SO_4$ and water. As 1M $(NH_4)_2SO_4$ appeared to have only a slight influence on the agglutination inhibition test, the two $(NH_4)_2SO_4$ eluates were reduced below this salt concentration by brief dialysis at 0–3°. The behaviour of the enzyme dialysed after exposure to similar concentrations of $(NH_4)_2SO_4$ in the absence of charcoal served as a control. There is an increase in the specific activity of the control material as a direct result of simple dialysis. The results show that when crude enzyme preparations which possess one H-unit associated with one mg. " tyrosine " are adsorbed in the presence of 3M $(NH_4)_2SO_4$ and eluted by 1M $(NH_4)_2SO_4$, half of the enzyme is recovered in the eluate and the material so obtained then shows

about 40 H-units per mg. " tyrosine." Table II indicates the degree of duplication obtained in consecutive experiments, the pairs of figures for specific activities showing good agreement.

TABLE II.—*Showing the Activity of Enzyme Preparations Eluted from Charcoal by Reducing the Ammonium Sulphate Concentration.*

Fraction tested.	Ammonium sulphate (M).	H-units per cent.	H-units/mg. tyrosine.
Original	0	(100)	1 ; 1
Control	3	60 ; 70	3 ; 3
Eluate A	2	6 ; 3	5 ; 4
Eluate B	1	38 ; 23	40 ; 40
Eluate C	0	6 ; 4	7 ; 8
Filtrate	3	10 ; 15	1 ; 1
SUM	—	60 ; 45	3 ; 3

The addition of 3 volumes of acetone to the crude filtrates as suggested by Schiff (1939), who employed a different medium, resulted in the deposition of an oily layer without giving rise to a significant increase in the specific activity of the material thrown out of solution. Treatment of fresh culture filtrates with an equal volume of acetone at 0° gave a satisfactory increase in purity with a yield of 70 to 80 per cent of the original activity. Methanol and ethanol have been employed with similar results. This method was used to prepare a quantity of the mixed enzymes suitable as a laboratory " standard." The details for preparing a useful quantity of enzymic material by this process are as follows :

Conical flasks filled nearly to the neck with 950 ml. of sterile peptone medium were inoculated and incubated overnight at 37°. " Superaid," 0·5 per cent, was added and the culture fluid filtered through paper. After cooling in an ice-salt bath the filtrate was mixed with an equal volume of acetone which had been cooled to − 30°. Regulation of the rate of acetone addition during the 10–15 min. required to complete the process ensured that the precipitation took place at 0°. Material thrown out of solution was collected by centrifugation and extracted with 25 ml. of cold McIlvaine buffer at pH 7·0. In six such experiments the average yield of the enzymes, purified some 150 times with respect to nitrogen or " tyrosine," per unit of activity, was not less than 60 per cent. Further extraction of the residue with buffer after the first treatment yielded additional material of lower specific activity. The main extracts were immediately shell-frozen in large round flasks and dried at 0·1 mm. pressure from the frozen state.

TABLE III.—*Showing the Activity of Acetone-Precipitated Blood Group Enzymes.*

Original (Av. A and H units/g.).	A-enzyme (units/g.).	H-enzyme (units/g.).	Depolymerase* (units/g.).	Nitrogen (mg./g.).
25	26†	24†	11†	70
25	1700	1200	450	31
36	1600	1600	640	30
48	2600	2400	440	22
58	2900	2900	500	18

* After storage for 3 months.
† Crude filtrate not acetone precipitated.

Thorough drying *in vacuo* over P_2O_5 of the white powders so obtained was followed by transfer to Macartney bottles at 0°. It was noticed, however, that not more than half the initial activity remained after two months' storage under these conditions. Details of the acetone precipitation of 4 culture filtrates are set out in Table III. The corresponding figures for a crude filtrate are also included. A carefully purified preparation contains at least 120 units of A- and H-activity per mg. nitrogen. Reprecipitation of material showing this order of activity with 0·4–1·3 volumes of acetone under the above conditions did not significantly increase the purity.

Immunization of Rabbits with Enzyme Preparations.

As a result of the failure to obtain preparations of A-enzyme devoid of power to inactivate H-substance by the methods described, an attempt was made to immunize rabbits with H-enzyme with the hope that an anti-H-enzyme would be produced which could be used to block the specific activity of H-enzyme without influencing the A-activity of the preparation. To this end rabbits were immunized with the unheated enzyme and with preparations heated at 56° for 10 min., which showed considerable specific A- and H-, and H-enzymic activity respectively.

The toxic nature of the material injected caused the death of four of the experimental animals, but one animal in each of the two groups survived the immunization and received a total of 1·6 mg. of enzyme protein over a period of 4 weeks. The natural A- and O-agglutinins were removed from the rabbit sera and suitable amounts of the sera were included in the standard test for enzyme activity. The results are given in Table IV, and show that on mixing the rabbit serum with the enzyme before the addition of substrate, some of the enzyme was neutralized or inactivated, for less enzymic activity was found than was added. Normal serum had no such effect.

TABLE IV.—*Showing the Neutralization of Enzyme by the Serum of Rabbits Immunized with* (a) *Heated and* (b) *Unheated Enzyme Preparations.*

Antigen (enzyme).	Antiserum used (ml.).	Units present.	Units found.
Heated	0·50	1·0A	0
	0·50	1·5A	0·5
	0·25	1·5A	1·0
	0·50	1·7H	0
Unheated	0·50	1·5A	0
	0·25	1·5A	0·8
	0·12	1·5A	1·2
	0·50	1·7H	0

The Enzymic Depolymerization of Pig Gastric Mucoid.

Specimens of mucoid isolated from commercial hog gastric mucin frequently show a high viscosity, and it has been observed that during the enzymic inactivation of the A and H serological properties of this material, a rapid fall in viscosity occurs. In our experience specimens of A- or H-mucoid isolated from individual pig stomach linings by peptic autolysis, and the A- and H-substances recovered from human ovarian cyst fluids, show no significant viscosity. Artificial

mixtures of A- and H-substances which individually show low viscosity are also non-viscous. It would seem, therefore, that a component of the commercial mucoid which is neither A- nor H-substance is responsible for the high viscosity of this material. It is of interest that the " depolymerase " present shows many properties which are similar to those of the group enzymes, and has been found to accompany these enzymes to the same extent after they have been purified as much as two hundred-fold in terms of the A- and H-enzyme activities (Table III). The highly viscous substrate is not depolymerized by hyaluronidase, and solutions of hyaluronic acid are not rendered less viscous by the blood-group enzyme preparations. Unlike hyaluronidase, the *Cl. welchii* depolymerase does not require optimum salt and buffer concentrations. The behaviour of buffered solutions of the depolymerase in the presence of the mucoid was followed in Ostwald pattern viscosimeters over a period of about 30 minutes at 37°. During this time a typical *Cl. welchii* (Type B) filtrate will reduce the relative viscosity of an equal volume of 0·25 per cent gastric mucoid from 1·6 to 1·3. The amounts of enzyme and substrate chosen were governed by the fact that at substrate concentrations greater than 0·25 per cent, the relationship between flow time and substrate concentration in the absence of enzyme ceased to be linear. The quantity of depolymerase present in the *Cl. welchii* culture filtrates decreased steadily after reaching a maximum at the end of 24 hours' growth. The pH-activity curve for this enzyme and the pH-stability range, which is considerably narrower than that of the blood-group enzymes, are shown in Fig. 4. Behaviour similar to that of the blood-group enzymes was noted on adsorption and precipitation, e.g. one-third of the depolymerase was recovered from culture filtrates by adding 70 per cent ammonium sulphate, the specific activity being increased 8 times, whereas the acetone purification procedure described above improved the specific activity some two hundred times. Hydrogen peroxide, potassium cyanide, and ascorbic acid, 0·02 M, caused some inhibition of activity. Toluene and merthiolate could safely be used as preservatives for the enzyme preparations, which were found to be stable for several weeks in the refrigerator.

DISCUSSION.

The action of the enzymes present in the culture supernatant fluids of certain strains of *Cl. welchii* (Type B) has been examined, using preparations of blood-group substances of animal and human origin as substrates.

The enzyme activities can be differentiated by heating the mixed enzyme preparations for 10 minutes at 55°, whereby the power to destroy the A and B serological character is lost. The capacity of the resulting solution to inactivate the H-substance is, however, unimpaired, and quite potent preparations of H-enzyme free from A- and B- activity can be obtained in this manner. Attempts to separate the enzymic activities by fractional precipitation techniques that involve the addition of ammonium sulphate, organic solvents or certain adsorbents to the crude culture filtrates have been, from a practical point of view, largely unsuccessful. Similarly, adsorption on charcoal columns failed to bring about a useful separation of the enzymes. By applying certain of these procedures, however, the specific activities related to total nitrogen or " tyrosine " were considerably increased. Nevertheless, no useful separation of the enzymes was attained, either from each other or from the depolymerase.

The properties of the enzymes most thoroughly studied are summarized in

Table V. It might be deduced from the results given there that the depolymerase could be freed from blood-group enzyme activity by dialysis, and that the depolymerase could be removed from the blood-group enzymes by allowing the preparations to stand at pH 11 for an hour at room temperature, but we have made no attempt to accomplish these changes.

TABLE V.—*Summarizing the Properties of the Blood Group Enzymes and Hog Mucin Depolymerase.*

Property.	A-enzyme.	H-enzyme.	Depolymerase.
pH optimum	5·5	6·5	6·8
Half activity, pH	4·9, 8·2	4·9, 8·3	5·6, 9·1
pH stability range	5–11	5–11	5·6–7·6
Temperature optimum	40–45°	40–45°	38°
Inactivation temperature (10 min.)	55°	60°	56°
Half activity, temperatures	25° 53°	25° 53°	33° 50°
Growth optimum (days)	1	1	1
Stability on dialysis	±	±	+
Stability on storage (liquid)	−	±	±
Average purification ratio (acetone)	200	200	200

The general lack of distinction between the physical properties of the blood-group enzymes is of interest. It could be surmised that certain groupings on the enzymic complex which are specific for the A- or B-substrate or responsible for the depolymerizing activity become denatured, leaving other groups unaffected and still able to bring about the inactivation of the H serological character. The evidence in support of such a conclusion is, however, far from complete. The inactivation by the enzyme preparation of the specific mucoids associated with the " Lewis " blood-group characters, Le^a and Le^b (Mourant, 1946 ; Andresen, 1947), has already been recorded (Grubb and Morgan, 1949).

The purified A-enzyme destroys the specificity of Group A mucoid when this character is measured by the haemolytic inhibition test, and in this property it differs from that shown by some crude enzyme preparations obtained from other types of *Cl. welchii* (Morgan, 1946) which failed to accomplish the destruction of A-specificity when measured by this technique. It is to be noted, however, that the differentiation of the two serological properties, the power to (*a*) inhibit iso-agglutination and (*b*) prevent the haemolysis of sheep-cells by rabbit serum, can be brought about by other means. Thus, it has been shown that after treatment with dilute acid the former activity is destroyed, whereas the latter activity is enhanced (Aminoff, Morgan and Watkins, 1948).

A strictly quantitative interpretation of the enzymic inactivation of the group substances in terms of the inhibition of agglutination is not straightforward when so little substrate remains, and when the indicator systems are relatively insensitive to changes in concentration of the reactants. The formula used to determine the units of activity when smaller or greater amounts than 94 per cent of the substrates are decomposed is based on the assumption that the reaction is not seriously affected by the almost complete destruction of substrate, and although primarily empirical, is of considerable practical value, and enables one to avoid making several titrations to determine the amount of the enzyme preparations required to bring about a lowering of the inhibition end-point by exactly four tubes.

Information about the chemical changes induced in the group active mucoids by the enzymes cannot readily be obtained when excessive amounts of crude enzyme material must be employed to destroy the serological character of these substances. The purified enzymes now described contain one-tenth as much nitrogen per unit as 1·0 mg. of the specific blood-group mucoid. They are, therefore, suitable for investigations of this kind and are being employed for this purpose. Similarly the action of the enzymes on the group specific character of stroma and intact erythrocytes is being studied.

SUMMARY.

The production from cultures of *Cl. welchii* of enzymes which destroy the specific serological characters of the human blood-group substances is described.

A quantitative method for measuring the activity of the enzymes has been applied to a study of their properties. The optimum pH values for the A- and H-enzyme activities are 5·5 and 6·5 respectively, and both enzymes are stable over the pH range 5–11.

A considerable purification of the enzymes was accomplished by the procedures described, and preparations containing 60 per cent of the original activity associated with 0·2 per cent of the crude filtrate nitrogen were obtained. These enzyme preparations, however, are not always stable when stored in the liquid or freeze-dried condition.

The serum of rabbits immunized with the purified enzymes developed weak anti-enzyme properties.

An additional enzyme which accompanied the group enzymes depolymerized an unidentified substrate in the "standard" preparation of hog gastric mucin. The enzyme is stable between pH 5·6 and 7·6, and shows an optimum activity at pH 6·8.

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