CCXIII. THE PROPERTIES OF ANTIGENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*

V. HYDROLYSIS AND ACETYLATION OF THE AMINO-POLYHYDROXY COMPOUND DERIVED FROM THE ANTIGEN

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In the preceding papers [Miles & Pirie, 1939, 3, 4] stages in the hydrolysis of the antigen of *Brucella melitensis* were described, the final product being a complex amine. This amine is prepared from its formyl derivative by hydrolysis. Considerable discoloration occurs when hydrochloric or sulphuric acids are used for the hydrolysis; if nitric acid is used there is no discoloration but a partly deaminated product results. The free amine can be obtained nearly colourless by refluxing a 2 % solution of AP, or better of the formylated amine (i.e. AP from which the phospholipin has been removed by very gentle acid hydrolysis), for 1 hr. with N H_2SO_4 and fractional precipitation with phosphotungstic acid. The first fifth of the phosphotungstic acid needed for complete precipitation brings down nearly all the colour; this precipitate is centrifuged off and the precipitation is then completed. The two precipitates are decomposed separately with baryta in the usual way and the excess of barium is removed exactly with sulphuric acid. The solution dries to a colourless scaly material.

It was stated previously [Miles & Pirie, 1939, 1] that AP contained 5·4 % N and 0·1–0·2 % S; recently, by working up preparations of AP that had been disintegrated with sodium dodecyl sulphate [Miles & Pirie, 1939, 2] preparations have been made that contain less than 0·1 % S and 6·0–6·2 % N. The amine that is derived from native AP contains less than 0·1 % S and less than 0·05 % P, for all the P in AP has been eliminated as phosphate and phospholipin. The other analytical figures were somewhat variable but fell in the following ranges: C 43–47; H 6·0–8·4; N 7·5–8·3 %.

AP has $[\alpha]_D^{16°}$ +43°; the free amine has $[\alpha]_D^{16°}$ +72° at about pH 9 and +61° when dissolved in $N/15$ H_2SO_4. This increase in specific rotation is of the order that would be expected on the hypothesis that the 25 % of phospholipin (PL 2) in AP contributes nothing to the rotation and that the loss of this and of the formic acid does not affect the asymmetric structures in the molecule.

In the Addendum to this paper Philpot describes the ultracentrifuging of both the amine and its formyl derivative. If we make the usual assumptions, the sedimentation constant of $0·95 \times 10^{-13}$ that he finds corresponds to a

minimum mol. wt. of 3300. The equiv. wt. on the other hand, whether calculated from the titration data given in the last paper or from the N content, is 170–190. In this paper some preliminary results on the hydrolysis of this high-molecular amine will be described.

Estimations of total sugar carried out by heating with orcin and H_2SO_4 [Pirie, 1936, 2] showed that AP contained 14 % carbohydrate estimated as glucose. The constituent responsible for this reaction is still present in the amine and, as would be expected from the removal of considerable amounts of non-carbohydrate material, the apparent carbohydrate content is now increased (20–22 %). The colour is developed rather more slowly in mixtures containing the amine than in control experiments containing the corresponding amount of glucose. Even after prolonged acid hydrolysis, e.g. 7 hr. at 100° with 5 N H_2SO_4, the "carbohydrate" content of what little amine can still be isolated by precipitation with phosphotungstic acid is 20 %. There is therefore good reason to look on this "carbohydrate" as an integral part of the amine molecule.

Reducing sugar estimations

Reducing sugar estimations have been carried out on hydrolysates by Somogyi's [1937] method, using glucose as the standard. The results of a typical experiment are given in Table I. If H_2SO_4 is used for the hydrolysis the destruction after the maximum reducing power has been attained is more marked, and fluids which have been decolored with charcoal after more than 2 hr. hydrolysis under these conditions give little or no reduction of the sugar reagent.

Table I

Time of hydrolysis at 100° with 3·6N HCl min.	Reducing sugar estimated as glucose %	Tintometer Red Units per mg. of amine
0	2·2	3
10	6·5	8
20	9·2	9·5
35	11·1	13·5
64	13·5	16
90	15·6	19
140	19·3	21·5
210	17·8	16
440	16·5	12

Glucosamine and 3-amino-glucose may be estimated by this method and it is reasonable to assume that other amino-sugars would be also. The low value of the reduction is therefore evidence that free amino-sugars in a quantity at all comparable with the N content of the amine either are not formed during the hydrolysis or else are not stable. Even after prolonged hydrolysis, when the reducing sugar value has begun to fall again, from a third to a half of the original amine can be recovered by phosphotungstic acid precipitation. This suggests that the destruction or modification of the hypothetical amino-sugar is more rapid than the hydrolysis of the amine. Evidence that the amine does contain an amino-sugar will be presented later when the products of deamination are described.

The extreme stability of glucosamine towards acid hydrolysis is well known, whereas the known amino-sugars that do not have the amino-group in position 2 are relatively unstable and either decompose, e.g. 6-amino-glucose [Fischer & Zach, 1911], or condense to a non-reducing anhydride, e.g. 3-amino-altrose

[Levene & Meyer, 1923; Bodycote *et al.* 1934]. Levene & Meyer found that this condensation was most marked when strong acid was used for the hydrolysis. Some hydrolyses have therefore been carried out with 0·2 *N* HCl; during the first 30 hr. at 100° the reducing sugar value, measured either by the Somogyi method or by the reduction of ferricyanide, rises and reaches 20 % but it does not rise further on further hydrolysis. Nitric acid has been found useful in the hydrolysis of pentosans [Heuser & Jayme, 1923]; when it is used for the hydrolysis of the amine discoloration is completely avoided but the yield of reducing sugar is no greater. After 4–5 hr. at 100° with 1·4 *N* HNO_3 the reducing sugar has risen to 21 % and on further hydrolysis it falls again. At this stage the amino-N content, reckoned on the dry weight, is less than 1 %. Nitric acid hydrolysis is therefore useless for a study of the hypothetical amino-sugar. Prolonged hydrolysis with 0·2 *N* HNO_3 was no more successful.

It is well known that such amino-sugars as have been tested do not give colours when heated with orcin and H_2SO_4 to an extent in any way comparable with the N-free sugars. It is of interest therefore to consider whether the 20 % of "sugar" that is found in the amine by the orcin method is due to a slight capacity of the nitrogenous component to react with the reagent or to the presence of 20 % of normal sugar. The question is still unsettled but 5–10 % yields of an as yet unidentified alcohol-soluble material containing 60 % of total and reducing sugar can be isolated from the supernatant fluids obtained after precipitating the unchanged amine from hydrolysates with phosphotungstic acid.

Amino-sugar estimations

Acetylated glucosamine or chondrosamine, after heating with dilute alkali, will give a red colour with Ehrlich's pyrrole reagent [Elson & Morgan, 1933; Palmer *et al.* 1937]. It is probable that this reaction is due to the formation of an oxazole ring during the alkali treatment [Morgan, 1938]. The behaviour of the hydrolysed amine towards this reagent is interesting for it gives the colour after treatment with alkali alone to a greater extent than after treatment with alkali and acetylacetone. Glucosamine, chondrosamine and also 3-amino-glucose (prepared by the method of Freudenberg *et al.* [1926]) give no colour if the acetylacetone treatment is omitted. AP, formylated amine and amine liberated by the minimum acid hydrolysis give no colour or, in the last case, very little whether they are acetylated or not. The amount of colour that is given after further hydrolysis varies both with the intensity of the hydrolysis and the period of heating with alkali. The former relationship is illustrated in Table I. Samples of a solution of formylated amine in 3·6 *N* HCl kept at 100° were withdrawn after the intervals stated and evaporated to dryness *in vacuo* over solid NaOH. The evaporation takes a few hours and the residue is nearly neutral. Solutions containing 5–15 mg. per ml. were then made up, the latter concentration being used for the less thoroughly hydrolysed material. 0·2 ml. samples were then heated with 0·5 ml. 0·5 *N* Na_2CO_3 for 30 min. in a boiling water bath. Alcohol and a solution of *p*-dimethylaminobenzaldehyde were then added as in the method of glucosamine estimation of Palmer *et al.* [1937]. The figures given are the intensity of colour in Tintometer units when the final volume is 4 ml. and the cell 1 cm. deep. Under the same conditions, but substituting a solution of acetylacetone for the Na_2CO_3, about a third of this amount of colour is given at the optimum but the colour does not fall off again after prolonged acid hydrolysis. The amount of colour given by glucosamine, etc. is not greatly affected by the time of heating with acetylacetone so long as the heating

is continued for more than 15 min. With the materials derived from *Brucella*, on the other hand, the colour goes on increasing with further heating with Na_2CO_3. Prolonged heating cannot be used quantitatively however for the fluid becomes discolored before the addition of Ehrlich's reagent.

From these results it is reasonable to conclude that one product of the hydrolysis of the amine is an amino-compound in some ways analogous to glucosamine. The results are compatible with the hypothesis that it is an amino-sugar with the amino-group in a position other than 2; on this hypothesis it is necessary to assume either that the amino-sugar is unstable towards acids or that it is easily converted into a non-reducing anhydride. As has already been stated, the isolation of apparently unchanged amine from solutions that have been hydrolysed so long that the reducing sugar value has passed the maximum is in favour of this hypothesis. It is probable therefore that it is merely a coincidence that the highest reducing sugar values that have been obtained are equal to the total sugar as estimated by the orcin method.

Hydrolysis under different conditions

Various other forms of hydrolysis have been tried both on AP and on the amine but the results have been no more satisfactory than those that have been described. 80 % acetic acid at 100° has little effect on the amine but reduces the serum precipitation end-point of AP by 90 % in 20 min., and there is a parallel fall in precipitability by $(NH_4)_2SO_4$. Some of the serological activity of AP remains after heating at 100° for an hour with $N/2$ NaOH but more vigorous hydrolysis destroys activity completely. There is, under these conditions, no liberation of inorganic phosphate and there is no precipitation of the phospholipin that we have referred to as PL 2 although PL 2 can be liberated by subsequent acid hydrolysis. Several attempts have been made to utilize the stability of AP towards alkalis in the extraction of the antigen or for separating it from agar but the method has not been found useful. On heating with alkali the free amine is gradually destroyed with the production of ammonia but no other product has been recognized.

Some attempts have been made to get identifiable products by hydrolysing the amine with strong H_2SO_4 in the cold. In one typical experiment 0·6 ml. of 70 % v./v. H_2SO_4 was added to 84 mg. of amine in 1 ml. of water and the solution was evaporated *in vacuo* over P_2O_5 in the cold. This technique was adopted because the dry amine dissolves very slowly in strong H_2SO_4. After 6 hr. it was diluted by adding ice and a solution of phosphotungstic acid was added; the precipitate, after decomposition with baryta, gave 65 mg. of apparently unchanged amine and a few milligrams of alcohol-soluble waxy material, similar to that already mentioned [Miles & Pirie, 1939, 4], was isolated from the supernatant. The process was repeated and after 18 hr. hydrolysis only 1/3 was still precipitable by phosphotungstic acid. After removal of the phosphotungstic acid from the solution with baryta a brown scaly material was left which could not be separated into components with appreciably different physical properties or chemical constitution by fractional precipitation with alcohol. It is not precipitated by the other agents such as picric, picrolonic or flavianic acids which precipitate the unhydrolysed amine. The total carbohydrate content (orcin) was 13 % and the reducing sugar content (Somogyi [1937], using glucose as standard) was 14 % without any further hydrolysis; the amino-N content was 4·5 %. There is therefore no evidence for the production in quantity of a reducing amino-sugar. By this technique hydrolysis proceeds without much discoloration but sufficiently far for the material to give the Elson & Morgan reaction after

treatment with Na_2CO_3. Under the conditions used in Table I it gives more colour, e.g. 40 Red Units per mg., than any other product that has been made from the amine.

Deamination of the amine

The amine can be conveniently deaminated under conditions similar to those of the Van Slyke amino-N estimation; the principal product of the reaction becomes insoluble in cold water after drying. From 46 mg. of amine dissolved in 0·3 ml. of water and 0·2 ml. of acetic acid and treated for an hour with 250 mg. of barium nitrite in 1 ml. of water, 50 mg. of a soft colourless flaky material were isolated by drying the fluid over NaOH after quantitative removal of the Ba with H_2SO_4. This material was washed three times with 2 ml. quantities of water on the centrifuge and both the solid and the washings were dried. The former weighed 40 mg. and the latter 10 mg.; each contained 50 % of carbohydrate by the orcin method but the soluble material gives the strong brown colour in the cold that is characteristic of ketoses and some partly oxidized sugar derivatives. A suspension of the insoluble deaminated material dissolves if kept on a water bath for 1–2 hr. giving an opalescent solution; on drying, the material reverts to the insoluble state. Deaminated material is more easily hydrolysed by acids than the amine and with nitric acid there is no discoloration. After 1 hr. with 0·2 *N* HNO_3 the reducing sugar value rises to 40 % and to 50 % in 2 hr.; after 4 hr. it is still 50 %. These results are the best evidence so far available that the amine contains material properly called an amino-sugar, for by deamination the amine, with a carbohydrate content of 20 % by the orcin method (which does not estimate those amino-sugars that have been tested), is converted into a slightly larger quantity of material containing 50 % of apparent carbohydrate.

Several preparations have been made but it has not proved possible to get a higher carbohydrate content than this. The results are substantially the same if the more alcohol-soluble parts of a preparation made by gentle hydrolysis, or if amine that has been vigorously hydrolysed, e.g. 2 hr. at 100° with *N* acid, are used. In these cases, however, little or none of the preparation is insoluble in water although some of it may take 5–10 min. to dissolve.

Deaminated material has the following analytical composition: C, 43–45; H, 6·0–6·3; N, 1·5–3·1 %. The preparations have never been N-free although they have been made from products in which all the N appeared to be in the amino-form both by titration and by Van Slyke estimation. Deamination reactions, however, seldom lead to a simple product and the same side reactions which lead to this residual N are probably responsible for the low total sugar content of the preparation. No products of the hydrolysis of the insoluble material have yet been identified but the particles give a strong Schiff test and no reaction for pentose.

Deamination of glucosamine

The deamination product from glucosamine, chitose, has been prepared for comparison with the material that has just been described. When prepared by exactly the same method, a water-soluble gum results but, as Levene & Ulpts [1925] have found, when a solution of this gum in dilute, e.g. *N* HCl is distilled *in vacuo* at 40°, a large part of the preparation becomes insoluble and resembles the deamination product from *Brucella* closely in its physical properties. Both the soluble and the insoluble parts of the preparation give colours with the orcin reagent; the former corresponds to a carbohydrate content of 40 % and the colour comes up quickly in the cold. The insoluble flakes, like those from *Brucella*, stain in a few seconds with Schiff's reagent. Chitose in the insoluble

state gives as much colour on heating at 100° with the orcin reagent as its own weight of glucose; at 55° chitose flakes give more colour than glucose and this colour is a clear red like that given by agar and the aldehydo-sugars [Pirie, 1936, 2]. This resemblance is of interest for there is evidence that chitose is a 2:5-anhydro-sugar [cf. Schorigin & Makarowa-Semljanskaja, 1935, for references] and that part of the galactose in agar is in the 3:6-anhydro-state [Percival & Forbes, 1938]. Because of these observations 3:6-anhydro-α-methyl galactoside[1] [Ohle & Thiel, 1933] was tested by the colour reactions described for agar and was found to give more colour under the conditions used than any other substance that has been tried. The argument put forward by one of us [Pirie, 1936, 1] that these colour reactions give support to the hypothesis that agar is partly composed of aldehydo-sugars must therefore be abandoned.

Acetylation and deacetylation of AP

Acetylation experiments have been carried out with materials at four different levels of hydrolysis: AP, formylated amine, amine and hydrolysed amine. The products of these acetylations are in many ways similar although they can be distinguished. Pennell & Huddleson [1937] have acetylated products which they call the endoantigens of *Br. melitensis* and *abortus* by heating with sodium acetate and acetic anhydride and they have raised the acetyl contents from 2·3 to 7·7 %. The chemical relationship between their endoantigen and the preparations that we have called [PLAPS] and AP is at present too obscure for it to be profitable to discuss the connexion between their observations and ours.

AP acetylates easily if it is precipitated from solution with alcohol, washed twice with pyridine and then suspended in a mixture of five parts of pyridine and two parts of acetic anhydride. The loose flocculent precipitate changes quickly into a tenacious gum and this dissolves in a few hours at room temperature. After incubation for 10 hr. at 38° the solution is distilled *in vacuo* to small volume and dried over H_2SO_4 and NaOH. The residue is soluble in acetic acid, pyridine and hot alcohol but not in chloroform or ether; it is partly soluble in water or cold alcohol but is easily precipitated by traces of salt. The fractions that can be prepared by adding water and salt to a strong pyridine solution do not differ in acetyl content but they are presumably aggregated to different extents. The total volatile acid [Elek & Harte, 1936] corresponds to 32–34 % of acetyl; this substance however still contains 7·5 % of formyl estimated by the method described in the preceding paper. The formyl therefore accounts for 11·1 % of the "acetyl" found, leaving 20·9–22·9 % of presumably real acetyl. The equiv. wt. of a substance containing 7·5 % of formyl is 387 and the acetyl content of a diacetyl compound of this equiv. wt. would be 22·2 %. There is evidence therefore that this gentle acetylation has introduced two acetyl groups for each formyl originally present. These are presumably *O*-acetyl groups for they can be removed by the Zemplen [1926] technique.

10 ml. of alcohol were added to a solution of 72 mg. of acetylated material dissolved in 0·4 ml. of pyridine; there was slight precipitation but the solution cleared on the addition of 1 ml. of 6 % NaOH in 90 % alcohol. After a few seconds it became turbid and a precipitate soon separated. This was centrifuged off after an hour and dialysed, 35 mg. being recovered. The regenerated antigen had a formyl content of 9·5 % and could be precipitated, like AP, by 37 % saturation with $(NH_4)_2SO_4$ at room temperature but, again like AP, a higher concentration

[1] The preparation was kindly supplied by Dr D. J. Bell.

was needed for precipitation at 0°. With antisera it precipitated specifically to an end-point of $1:1 \cdot 4 \times 10^6$; this end-point compares favourably with the $1:2 \cdot 5 \times 10^6$ that is characteristic of good preparations of AP [Miles & Pirie, 1939, 2].

Acetylation of the degradation products of AP

The other three substances have been acetylated by adding 15 parts of pyridine to a 20–30 % solution of the amine or formylated amine in water and then 10 vol. of acetic anhydride. Formylated amine remains in solution after the addition of the pyridine but the other two precipitate; they all precipitate on the addition of the acetic anhydride. Amine and formylated amine dissolve completely at room temperature after a few hours whereas amine which has been vigorously hydrolysed, e.g. 2 hr. at 100° with 2 *N* HSO_4, may need 12 hr. at 38° before all is in solution. After 24 hr. at 38° they are dried as before. All three dissolve in alcohol, pyridine and acetic acid but only acetylated hydrolysed amine is appreciably soluble in chloroform; they are all insoluble in ether. It has already been pointed out that the antigen in its various stages of disintegration is more soluble in cold ammonium sulphate solution than in warm solutions of equal salt content. These acetylated products behave in a similar manner. They dissolve slowly in cold water to give clear solutions but on warming the solutions become turbid and will precipitate in time. The process is perfectly reversible on cooling and can readily be demonstrated with a 1–2 % solution.

Acetylated formylated amine contains the expected 10–11 % of formyl whereas the others contain none; the percentages of total volatile acid, reckoned as acetyl [Elek & Harte, 1936], are 36 %, 31 % and 31 % respectively with the three substances. The formylated amine has therefore still got two acetylatable groups besides the formylated amine group, but after hydrolysis one of these appears to be lost, for the diacetylated derivative of a substance which, like the amine, has equiv. wt. 180 would contain 32 % of acetyl. The apparent acetyl content is raised neither by further periods of acetylation with pyridine and acetic anhydride nor by hydrolysing for 3·5 hr. during the acetyl estimation instead of for the 2·5 hr. recommended by Elek & Harte.

N-acetylated amine and *N*-acetylated hydrolysed amine are more easily hydrolysed by acids than the free amine if hydrolysis is measured by the production of reducing sugar. A typical experiment carried out with a 0·5 % solution of *N*- and *O*-acetylated hydrolysed amine in 0·8 *N* HCl at 100° gave the following reducing sugar values (Somogyi) after 15, 30, 60, 133 and 196 min.; 6·4, 13, 19·5, 25·5 and 26 %. It is clear that, although reducing sugar is produced more quickly under these conditions than under those in Table I, the final values are so nearly the same that little would be gained by using acetylated material in the study of hydrolysis of the amine. These results are in agreement with the interesting observation of Moggridge & Neuberger [1938] that *N*-acetyl methyl glucosaminide is more easily hydrolysed, with the production of reducing sugar, than methyl glucosaminide itself. In both cases the effect is only marked during the first stages of hydrolysis because hydrolysis proceeds both at the glucoside linkage and at the *N*-acetyl group. Free glucosamine is very stable in the presence of acids and Moggridge & Neuberger obtained an 80 % yield of reducing sugar. With the acetyl derivatives of the much less stable amine from *Brucella*, on the other hand, the highest value has been 26 % and further hydrolysis leads to the destruction that has already been described. Unsuccessful attempts have been made to hydrolyse *O*-acetylated *N*-formylated amine with formic acid,

but with 90 % acid, although there is little or no liberation of amino-N, destruction is more rapid than the production of reducing sugar; the course of hydrolysis with dilute formic acid is similar to the course with hydrochloric acid.

Acetolysis

It was hoped that stable and recognisable breakdown products would result from the acetolysis of these acetylated substances, but the yields of the only crystalline derivative that has been isolated from several attempts at the acetolysis of acetylated amine and *O*-acetylated *N*-formylated amine have been too small for any attempt at its identification.

To 40 mg. of acetylated amine, dissolved in 0·2 ml. of acetic acid, 1 ml. of acetic anhydride was added. All remained in solution although these substances do not dissolve if acetic anhydride is added to the solid. The solution was cooled and 1 ml. of acetic anhydride, to which 0·3 ml. of H_2SO_4 had been added with cooling, was poured in, causing the separation of resinous masses which soon dissolve. The mixture was left at 38° for a week by which time it had become brown. Nothing seems to be gained by using stronger acid or by leaving the mixture for longer at 38° in the first instance. It was poured on to a mixture of ice and 4 ml. of 2*M* sodium acetate solution and, after a few hours, extracted 3–4 times with its own volume of chloroform. The aqueous part was dried and extracted with pyridine but little or nothing was found in the extract. On evaporation the chloroform extract weighed 18 mg. and this material was extracted repeatedly with cold ether. The extracts, on evaporation, were partly crystalline and this crystalline material, weighing 1–3 mg., could be separated from the remainder by dissolving it in a small quantity of ether. It crystallised well from dilute alcohol after it had been separated from uncrystallizable material in this way. A pool of products from several experiments of this type was analysed and found to contain 3·9 % N (Dumas); 32 % acetyl and 12 % carbohydrate (orcin).

The part of the acetolysis products that is extracted by chloroform but is not soluble in ether will, on further acetolysis, give more of the ether-soluble material if the same technique is adopted. A more detailed study of this substance will be undertaken when more of the antigen is available.

Discussion

On account of the elaborate series of partial hydrolyses, each followed by a fractionation, by which this amine has been derived from the bacterial antigen, itself a thoroughly fractionated product, it is reasonable to assume that the amine is as likely to be pure as any other molecule of colloidal dimensions that has been studied. Philpot's surprising observation that the product of these actions is centrifugally homogeneous lends further valuable support to this assumption. No products of the hydrolysis of the amine have yet been identified and it would seem that the methods that we have used in this work are unsuitable for the study of a molecule of this type for the losses on hydrolysis have been too great for useful results to be got with the quantities of material at present available. There is, however, reason to think that the amine is built up from only two or three different units and that, since its mol. wt. is only of the order of 3300, it should be possible soon to determine its structure.

Three facts have been established; the presence of 7·5–8·3 % of N all, or nearly all, in the amino-form; the presence of two hydroxyl groups for each N atom in preparations at the penultimate stage of hydrolysis; the presence of

material that reacts as carbohydrate by the orcin method. A polysaccharide built up from amino-hexose units would, if we neglect the small effect of the molecule's ends, contain 8·7 % of N, two hydroxyl groups for each N atom and would give no colour with the orcin reagent if we judge by those which have been studied. If the amine gives the orcin colour owing to the presence of a hexose in the molecule in the proportion of 1 hexose to 4-amino-hexose units it should, if it is a simple linear molecule and we neglect ends as before, contain 6·9 % of N and have three hydroxyl groups for each N atom. This hypothesis can therefore be excluded. Of the many other possible hypotheses two merit consideration; that the orcin colour is due to a substance smaller than a hexose, e.g. a triose, or that the amino-sugar from which the amine is apparently built is itself responsible for the action. This last hypothesis is in many ways the most attractive.

Summary

The main antigen of *Brucella melitensis*, the amine and the *N*-formylated amine derived from it, have all been *O*-acetylated. The properties and interrelationships of these substances is discussed.

The products of acetolysis of *N*- and *O*-acetylated amine are described.

The course of hydrolysis, measured by the production of reducing sugar, of the amine, of its *N*-acetyl derivative, and of the product of its deamination by nitrous acid are described, and the properties of the deaminated material compared with those of deaminated glucosamine.

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ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

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(with technical assistance by E. Dodwell)

Five specimens from *Brucella* were sent by Mr Pirie. They were examined in 0·5–0·95 % solution in 0·2*M* NaCl plus 0·01*M* phosphate of *p*H 7, in a Svedberg oil-turbine ultracentrifuge, by the "diagonal schlieren" method. The refractive increment of the sedimenting material was determined by integrating the curves on the original prints with the aid of a planimeter. As the areas are rather small for this purpose the tracing point of the planimeter was fitted with a lens, and the wheel was placed on a "Perspex" surface which gives perfect smoothness without slip. A Stanley "Allbrit" planimeter was used, set at 4:1 cm.^{2}, and the area was traced five times for each reading. Used in this way tests showed that the planimeter was accurate to 0·01 cm.^{2} The base-line was chosen by drawing a straight line between two points on either side of the sedimentation boundary. In all runs so far studied, whatever the substance, the area has decreased markedly after the edge of the boundary has entered the lower half of the cell. We have evidence that this is probably due to convection, so we now base our determinations of refractive increment on the means of the earlier photographs. Tests with a specimen of serum albumin made by Dr Ogston gave with this procedure an area 99 % of the theoretical. With smaller or less homogeneous molecules the accuracy is less, but in our experience the extra run required to give the true base-line is seldom justified.

Table I

1 Photograph	2 Prep. no.	3 Material	4 S_{20} 10^{-13}	5 %	6 Homogeneity
Meniscus	317A	AP, as usually prepared	*ca.* 20	*ca.* 65	Very poor, many faster particles
	317B	AP disaggregated by treatment with dodecyl sulphate	12·3	78	Good
Meniscus	317E	317B reaggregated by precipitation with ammonium sulphate	*ca.* 50	*ca.* 57	Very poor, many faster particles
	346J	"Free amine"	0·95	50	Fair
	347A	"Formyl derivative of amine"	1·02	100	Good

Table I summarizes the results. Col. 4 gives the sedimentation constant in units of 10^{-13}. The figures in col. 5 are the refractive increment of the observably sedimenting material expressed as a percentage of the total refractive increment. The homogeneity given in col. 6 is only a rough guess and is decided by inspection of the photographs allowing qualitatively for the diffusion to be expected of a substance having the observed sedimentation constant. The photographs of 317A and 317E are complicated by various accidental factors and have been omitted.

The general conclusion from Table I is that the breakdown-products 317B, 346J, and 347A are more homogeneous than the original material 317A or the reaggregated material 317E. This is analogous to the fact that casein becomes homogeneous if the calcium is removed [cf. Philpot & Philpot, 1939]. The material not accounted for in col. 5 may be too low-molecular to sediment, or it may be high-molecular but so heterogeneous that its sedimentation curve is indistinguishable from the base-line.

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REFERENCE

Philpot, F. S. & Philpot, J. St L. (1939). *Proc. roy. Soc.* B, **127**, 21.