CCXII. THE PROPERTIES OF ANTI-GENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*

IV. THE HYDROLYSIS OF THE FORMAMINO LINKAGE

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THE predominating antigenic substance present in smooth strains of *Br. melitensis* has been isolated and described in some detail; it appears to be a complex of some phospholipins, a protein-like substance and the formyl derivative of a poly-hydroxy amine [Miles & Pirie, 1939, 1, 2]. This complex "native" antigen may be successively deprived of the phospholipins and the protein-like substance. The formamino compound which remains is apparently fully antigenic and retains the serological activity of the native antigen. At this level of complexity the antigen was for convenience called AP. After precipitation with ammonium sulphate it has mol. wt. about 1,000,000 which may be reversibly reduced to 100,000–200,000 by disintegration with sodium dodecyl sulphate. Hydrolysis of AP in *N*/10 HCl for 5–10 min. at 100° liberates another phospholipin (PL 2) and partially liberates an inorganic phosphate; in 45–60 min. the phospholipin is coagulated and the phosphate fully liberated [Miles & Pirie, 1939, 3]. After this much hydrolysis the formamino compound is still retained by a cellophane membrane, but it will no longer precipitate with homologous antisera.

Many modifications of the method of preparing this phospholipin-free formamino compound have been tried. The method already published seems to be the most convenient although there is some liberation of amino groups during the hydrolysis. The resulting mixture of formylated amine and partly deformylated material can be fractionated to some extent either by dialysis or by precipitation with alcohol in the presence of salts. After these treatments the less readily diffusible material and the more readily precipitable materials have been the best preparations obtainable. By drying frozen solutions the formylated amine is obtained as a fluffy product which is readily soluble in water, and which should contain less than 0.6% amino-N (Van Slyke). AP, the complex of formylated amine and phospholipin, on the other hand, dissolves in water with difficulty if it has once been dried, and contains no detectable amino-N. In this paper the further stages in the hydrolysis of the formylated amine will be described.

Formic acid has been identified as a product of the hydrolysis of AP by a number of different methods and we have produced evidence that it is linked to an amino group. The question has been studied in some detail for formyl derivatives have not, apparently, been described before in nature if we exclude substances such as chlorophyll *b* [Fischer & Breitner, 1935] and *Spirographis haemin*

[Fischer & von Seemann, 1936] which, although described as formyl derivatives, are simply aldehydes. Formic acid is, of course, well known in the free state, e.g. in ants and in fruit juices, and in small quantities it has often been recognized in the products of vigorous hydrolysis of organic materials by acids and alkalis [e.g. Sundwik, 1881]. Adenine [Stevens, 1937] and lycoperdin [Kotake & Sera, 1913] are peculiar in giving high yields of formic acid on hydrolysis but only under conditions of a different order of intensity from those used with the *Br. melitensis* amine. Lycoperdin, which is isolated from the acid hydrolysate of puff balls, on hydrolysis yields glucosamine as well as formic acid, but the amino groups appear to be freed at a level of hydrolysis below that required to liberate the formic acid.

The fact that formyl groups have not been found more often in nature cannot be taken as evidence that they are in fact rare, for the identification of volatile acids is often not very conscientiously carried out. It is probable that some of the "acetyl" groups that have been described are in fact formyl groups.

Identification of formic acid

Quantitative estimation of the volatile acid liberated on hydrolysis can be carried out by the method of Elek & Harte [1936]; by this method 1 equiv. of acid is given by 270–300 g. of AP and, if this is reckoned as formic acid, it corresponds to 9.5–11 % of formyl groups. In this estimation *p*-toluenesulphonic acid is used for the hydrolysis, but if one wishes to work up both the base that remains in the distillation flask and the volatile acid, sulphuric acid has obvious advantages, although with this acid recovery cannot be quantitative since the mixture cannot be distilled to dryness. 2 ml. 2*N* H₂SO₄ were used for the hydrolysis of 100–700 mg. quantities of AP or formylated amine. After refluxing for 30 min. the mixture was allowed to distil with the addition of water to keep the volume constant. About half the volatile acid comes over in 30 ml. of distillate and there is some charring of the materials in the distillation flask. The distillate was neutralized with 0.36*N* Ba(OH₂), concentrated to 2 ml., centrifuged from a trace of solid and allowed to crystallize in a desiccator. It was clear, from microscopical examination, that this product was slightly contaminated with gum. The latter could be removed by recrystallization from water or, more conveniently, from dilute alcohol. More crude Ba salt could be isolated from the mother liquors making the total yield 75 % of the original weight of the salt; it is clear therefore that this salt is the principal component of the neutralized distillate. The gummy residue still contained about 50 % of barium formate, as estimated by the mercuric chloride technique; its other constituents have a much higher carbon content but they have not been studied further.

Several samples of Ba salt, recrystallized from water, have given the following analyses (Weiler): C, 11.32; H, 1.08; Ba, 60.3 %. The C and H figures are appreciably higher than the theoretical (C, 10.6; H, 0.8; Ba, 60.2 %), but a sample of barium formate, prepared from Kahlbaum's formic acid and analysed by the same analyst at the same time gave similar results. Furthermore, the figures differ widely from those for any other fatty acids; the other acids which would give Ba salts with such high Ba and low C contents are non-volatile or give Ba salts that are not soluble in water at neutrality.

The formic acid which remains in the flask after distillation can be recovered by extracting the acid fluid several times with methyl ethyl ketone for, as Archibald [1932] has shown, the partition coefficient is 1.3 and is, therefore, greater with this solvent than with the others that have been tested. Barium formate is prepared from this extract by counter-extraction with enough aqueous

$\text{Ba}(\text{OH})_2$ to make the mixture alkaline; the aqueous layer is worked up as before. This fraction is microscopically purer than that made from the distillate but, after recrystallization, it gives the same analytical figures. The total yield of barium formate from these two fractions is 70 % of what would be expected from the N content of the starting material, e.g. 164 mg. of formylated amine containing 6.5 % N gave 61 mg. of barium formate. In some other experiments the distillates were neutralized, or the methyl ethyl ketone extracts counter-extracted, with aqueous NaOH and dried. Lead formate was prepared from the crude sodium formate by adding the corresponding amount of lead nitrate solution and evaporating if necessary. Lead formate is sparingly soluble and crystallizes well and is therefore convenient for the characterization of the acid, but the isolation of lead formate cannot easily be combined with a study of the other components of the distillate or extract. After recrystallization it gave the following analytical figures: found: C, 8.3; H, 0.87 %. Calculated: C, 8.08; H, 0.674 %.

Distillates from many hydrolysed bacterial fractions have been tested for the presence of acetic acid by the colour reaction with lanthanum salts and iodine [Kruger & Tschirch, 1930]. Formic acid gives a yellowish opalescence with this reagent but the blue colour due to acetic acid can readily be detected in its presence. Acetic acid has never been detected in preparations which had not been artificially acetylated and we have never had reason to suspect the presence of large amounts of any other volatile acid. Pennell & Huddleson [1937] found a fraction in *Br. melitensis* which, after saponification, gave a yield of volatile acid corresponding to an acetyl content of 48 %; we have never encountered such a fraction.

The evolution of CO on incubation with H_2SO_4 is a characteristic property of formic acid and the formates. The action proceeds quickly at 37° and may conveniently be followed in the Warburg manometer. 3 ml. of conc. H_2SO_4 are put in the main chamber of the manometer and dry barium formate in the side bulb; after equilibration the two are mixed.

In one experiment with 1.04 mg. of barium formate the pressure rose to 142 mm. of Brodie's fluid in 10 min., to 159 mm. in 20 min. and to 160 mm. in 25 min. The manometer used had a volume of 16.9 ml. Since the correction for the solubility of CO in H_2SO_4 is small it is legitimate to use the value 0.024 which Christoff [1906] found for the solubility coefficient in 95.6 % H_2SO_4 . The reaction was carried out at 37° and 764 mm.; the weight of CO liberated is therefore

$$\frac{28 \times 160 (13.9 + 0.024 \times 3) \times 273 \times 760}{100,000 \times 22.4 \times 310 \times 764} = 0.251 \text{ mg.}$$

This is 102 % of the theoretical value. By the same technique commercial samples of barium formate have given 95–105 % of the expected pressure rise.

Formic acid is often estimated by titration with potassium permanganate. Barium formate made from the antigen gives satisfactory results which will not be recorded since the method has little specificity.

The formamino linkage

In the course of determining the mode of linkage of formic acid it was necessary to compare the rates of production of formic acid and of amino groups during hydrolysis; for this purpose the acid distillation method is clearly unsuitable but a method based on the extraction of the acid with methyl ethyl ketone was convenient. Small amounts of methyl ethyl ketone do not interfere with the standard and apparently specific method of estimation based on the reduction of mercuric to mercurous chloride; 1 ml. quantities of a 1.0 % solution

of formylated amine in 0.33*N* HCl were withdrawn after various periods of heating in a boiling water bath and shaken vigorously with two 5 ml. quantities of methyl ethyl ketone. The combined extracts were mixed with 0.5 ml. of 0.5*N* Na₂CO₃, evaporated to dryness on a water bath and neutralized or slightly acidified with acetic acid. 10 ml. of a saturated solution of mercuric chloride containing 2% of acetic acid were added and the mixture heated in a stoppered 50 ml. flask in a boiling water bath for 2 hr. The calomel was filtered off on a small, weighed, sintered glass funnel, washed with *N* HCl, water and alcohol and weighed after drying at 100°. Control experiments with known quantities of formic acid showed that the reduction proceeded quantitatively with 0.5–2.0 mg. of acid and that formic acid was 75–80% extracted by the two treatments with methyl ethyl ketone. In Table I the results of such an experiment are recorded and also the

Table I

1 Time of heating min.	2 Amino-N %	3 Formic acid found i.e. wt. of Hg ₂ Cl ₂ × 10.3 %	4 Formic acid found expressed as % of the amount of formic acid corresponding to the amino-N in col. 2
0	0.8	0	—
10	2.8	6.7	73
15	3.7	9.6	79
40	4.8	13.2	83
120	6.6	16.4	76

The recovery of formic acid by the method used is 75–80%.

amino-acid content of the fluid after various periods of hydrolysis. It can be seen that the formic acid and amino groups are liberated at similar rates and it is, therefore, reasonable to assume that the starting material was an *N*-formyl derivative. Experiments of this type cannot be carried out satisfactorily with unhydrolysed AP, for the PL2 that is liberated during the hydrolysis interferes with the separation of the two liquid layers.

The deformylated compound

Solutions of AP from which the phospholipin components, the phosphate and the formyl group have been removed react with nitrous acid under the same conditions as α -amino-acids and glucosamine. Using the usual Van Slyke technique a volume of gas is evolved which corresponds to 90% of the total N (Dumas). This agreement is satisfactory but, although glucosamine itself behaves like an amino-acid under these conditions, other amino-sugars and related substances have been found to give anomalous results [Levene & Meyer, 1923]. The amine, but not the formylated substance, also gives a "ninhydrin" reaction as strong as that given by the same weight of glucosamic acid or leucine and for this reason it was at first thought that the hydrolysate contained an amino-acid. That this is not the case is clear for two reasons: no CO₂ is evolved when the material is boiled with ninhydrin under the conditions used by Van Slyke & Dillon [1938] although a deep blue precipitate is formed; also, there is in the hydrolysate no titratable group with a *pK* in the usual range for carboxyl groups, i.e. between 5 and 2.

Titration curves have been plotted, using a double hydrogen electrode system [cf. Pirie & Pinhey, 1929], for AP, formylated amine and free amine. In an earlier paper [Miles & Pirie, 1939, 1] we stated that AP, which had been precipitated by alcohol from acid solution and then washed thoroughly with alcohol, behaved like

an acid with equiv. wt. 8000. This has been confirmed by comparing the titration curve of a 1.5% solution of AP with that for the same volume of water. Maximum buffering occurs between pH 3 and 4. As hydrolysis proceeds a new group with pK at 6.8 appears and the amount of acid used in titrating this group is roughly proportional to the amino-N content of the preparation, e.g. a sample of formylated amine with 0.6%, or less, amino-N had equiv. wt. 2000 and one with 1.3% had equiv. wt. 950, whereas preparations from which the formic acid had been completely hydrolysed had about 7% amino-N and equiv. wt. 186. Although it is clear that glucosamine itself is not a constituent of AP, its titration curve was plotted for comparison since, so far as we are aware, the dissociation constants of the amino-sugars have not hitherto been measured. The observed value for pK_a at 19° for a 0.033*M* solution in the presence of 0.037*M* NaCl was 7.8. This value suggests that glucosamine might be useful as a buffer in the neutral range with certain systems.

Comparison of the amine and its formylated derivative

Alcohol will precipitate AP from solution and, with more difficulty, the formylated amine also. The free amine in neutral or alkaline solution is not easily precipitated, but its salts, especially the sulphate, separate as heavy oily layers when alcohol is added in small quantities to their strong aqueous solutions. Other solvents, such as methyl ethyl ketone, have the same effect. The separation of this oil causes difficulty in the removal of formic acid from hydrolysates unless dilute solutions are used. With other precipitants there are similar differences between the free amine and its formyl derivative. Phosphotungstic acid will only precipitate AP and the formylated amine completely from strongly acid solutions (e.g. 0.5*N* H₂SO₄) and the precipitates dissolve easily in water when washed. The free amine, on the other hand, can be precipitated from only slightly acid solution and the precipitate can be washed with water. For complete precipitation the amine requires about six times its weight of phosphotungstic acid whereas the other materials are precipitated by half this quantity. It is interesting to note that lycopodin which, as has been stated, yields glucosamine on hydrolysis, is also precipitated by phosphotungstic acid although glucosamine itself is not. Picric, picrolonic and flavianic acids precipitate aqueous solutions of the amine but do not precipitate the formylated amine or AP. These precipitates are all gummy and amorphous although the third gives a birefringent gum which can be orientated by shearing. Attempts have been made to get crystalline salts with a large number of acids but so far they have been unsuccessful.

The presence of material with a high C content in the crude preparations of barium formate, made by neutralizing the distillates from hydrolysates, has already been mentioned. A similar material is left when the methyl ethyl ketone solution that remains after counter-extraction is evaporated. These substances account for less than 5% of the formylated amine, but we have no evidence that the yield is constant or that they are primary hydrolysis products of the molecule. It is probable, however, that they are formed or liberated during the hydrolysis, for they dissolve in the ordinary organic solvents whereas nothing can be extracted from AP or formylated amine by those solvents.

Solutions of the amine and its formyl derivatives are not precipitated by homologous *Brucella* antisera. Nevertheless, the various fractions isolated from hydrolysates of AP may be compared serologically by measuring their capacity to inhibit agglutination of *Br. melitensis* by a monospecific serum. The minimum concentrations producing a standard degree of inhibition are listed in Table II.

Table II. *Specific inhibiting titres*

Starting material	Nature of solution tested	Inhibition titres (expressed as conc. of starting material)
1. AP (gentle hydrolysis)	(a) AP	1: 70,000
	(b) AP partly split into phospholipin and formylated amine	1: 40,000
	(c) AP completely split into phospholipin and formylated amine	1: 12,000
2. Dialysed preparation of formylated amine	(a) Formylated amine	1: 12,000
	(b) Amine	1: 250
	(c) Partly hydrolysed amine	> 1: 50

The inhibition titre of AP in Table II is about four times greater than that already recorded for AP [Miles & Pirie, 1939, 2], for, although the same method of titration has been used, the end-points are in this case taken as the concentration reducing the degree of agglutination by 50 %. This end-point can be determined with greater accuracy than the one previously measured, namely, the concentration producing total inhibition.

The inhibiting power of AP appears to depend in part on the integrity of the complex of formylated amine with the phospholipin PL2. Complete conversion of AP into a suspension of phospholipin in a solution of the amine reduced the titre to 1 : 12,000. If this residual inhibition is due to the free formylated amine in the mixture, which contains about 20 % by weight of the phospholipin, the inhibiting titre of the formylated amine alone should be in the region of 1 : 15,000. The observed figure of 1 : 12,000 (Table II, 2 (a)) is sufficiently close to warrant this assumption. Deformylation results in a big reduction of inhibiting power, and hydrolysed amine is inactive in concentrations as large as 2 %. Inhibiting power is reduced progressively with the loss of phospholipin or formyl groups and with destruction of the amine. Though it is likely that these three chemical structures are the important antigenic determinants of AP, it is impossible on the available evidence to exclude the existence of an unrecognized constituent which is progressively liberated in the course of a hydrolysis whose stages are independently defined by the loss of phospholipin and the formyl groups.

In the following paper some preliminary conclusions on the constitution of the high-molecular amine which results from this hydrolysis will be presented.

SUMMARY

Formic acid has been identified as a product of the hydrolysis of the antigen of *Brucella melitensis* and evidence is presented that the unhydrolysed antigen contains a formamino group.

Some chemical, physical and serological properties of the high-molecular amine are compared with those of its formyl derivative.

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