Studies upon the Gram Reaction of the Basiphil Cells of the Anterior Pituitary

Part II. Observations upon the Effects of Various Methods of Fixation

By R. R. WILSON AND C. L. FOSTER

(From the Departments of Morbid Anatomy and of Biology, St. Mary's Hospital Medical School, London)

With one plate (fig. 1)

SUMMARY

To investigate the effects of fixation on the Gram reaction in the pituitary basiphil granules, frozen sections were made from fixed and unfixed glands, paraffin sections from frozen-dried glands, and paraffin sections in the usual way from whole glands, as well as from several pieces of glands embedded in one block. The fixatives were various well-known mixtures and single substances. Staphylococci were also studied, after treatment with some of the fixatives.

The basiphil granules were always Gram-positive after fixation in formaldehyde-saline, formaldehyde-Zenker, Zenker's fluid, and Zenker stock-solution. Frozen sections of unfixed glands, and sections of frozen-dried glands, were always Gram-negative; but such sections were usually Gram-positive if left in formaldehyde-saline for at least 12 hours before staining. All other fixatives studied gave negative results. The staphylococci were always Gram-positive.

These findings, and some questions arising from them, are briefly discussed.

INTRODUCTION

IN a recent communication (Foster and Wilson, 1952) we reported the first results of a study of the Gram-positive properties of the cells of the human anterior pituitary. It was found that the Gram-positive reaction occurred regularly in the cytoplasmic granules of the basiphil cells, but never in those of the acidophils, nor in the cytoplasm of the chromophobes. The Gram-positive reaction in the basiphils was not abolished by ribonuclease, though it was abolished by hot oxygenated bile salt, after the method of Henry and Stacey (1946). Moreover, the distribution of ribonucleic acid, as shown by pyroninophilia and ribonuclease extraction, did not correspond with the distribution of Gram-positive staining. For these and similar reasons we concluded that it was unlikely, or at least unproven, that ribonucleic acid was the essential factor in determining the Gram-positive reaction of the basiphil granules.

These results were obtained from human post-mortem material, which was fixed in 10 per cent. formaldehyde-saline, embedded in paraffin, and sectioned

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in the usual way. We pointed out that this method differs from that of most workers studying the Gram reaction in bacteria, whose material is fixed by heat. We decided accordingly to investigate the effects of various methods of fixation. The purpose of the present paper is to record the results of this work.

Materials

Human pituitaries obtained at post-mortem have again been used; specimens fixed more than 12 hours after death have been rejected, those fixed less than 6 hours after death have been specially sought. We have also used pituitaries from the rabbit, rat, and monkey (macaque); and we made a few observations on staphylococci.

Methods

As our principal object was to study the effects of various fixatives on the Gram reaction, it was necessary—

(i) to exclude as far as possible the effects of reagents used in embedding and sectioning, which was done by making frozen sections of fixed and unfixed glands, and in two cases (rabbit) by the freezing-drying technique;

(ii) to keep embedding and cutting processes, and the actual Gram staining, as constant as possible, which was done by embedding a number of specimens in a single paraffin block.

The details of the methods now follow:

Human pituitaries were either halved horizontally or cut into more numerous pieces by antero-posterior and transverse sections.

(*a*) Halved pituitaries fixed in formaldehyde-saline were sectioned on the freezing microtome, the sections received in distilled water, mounted on albumenized slides, and stained.

(*b*) Halved unfixed glands were treated in the same way.

(*c*) Halved unfixed pituitaries were sectioned on the freezing microtome, the sections received in formaldehyde-saline and kept in it for varying periods, mounted on albumenized slides, and stained.

(*d*) Two rabbit pituitaries were embedded in paraffin by the freezing-drying technique, the sections cut in the usual way (some being kept in formaldehyde-saline for varying periods), mounted and stained without denaturation in alcohol.

(*e*) Halved human pituitaries, or whole animal pituitaries, were fixed in various fixatives, embedded singly in paraffin, sectioned and stained as usual.

(*f*) Pituitaries were cut with a razor-blade into pieces, up to ten in number. The pieces of each gland were then fixed separately in different fixatives, and after 12–24 hours were taken side by side through the various stages of paraffin infiltration and finally embedded in a single block of wax. Sections

were cut from this so that all the pieces were represented on the same slide.

(*g*) A few 24-hour cultures of staphylococci were obtained. Suspensions of each culture in water were prepared. Ten films were made of the suspension of each culture, in the ten compartments formed on a slide by grease-pencil lines; the films were allowed to dry in the air. Each of the ten films was then covered by a drop of one of ten fixing reagents (formaldehyde-saline and the nine 'single substances' listed below), which were left in position for 5 minutes to overnight, with precautions against drying; the fixatives were finally washed off with water. Films of each suspension were also fixed by heat.

(*h*) The fixing reagents used fall into two groups: (i) mixtures, (ii) single substances.

(i) The mixtures were: 10 per cent. neutral formaldehyde-saline (included among 'mixtures' because Crawford and Barer (1952) have shown that the saline has its own effect). Zenker's fluid. (The 'Zenker' made up in the Biology Department contains no sodium sulphate; we have not observed any difference in the results.) 'Zenker stock' (Zenker's fluid without acetic acid). Formaldehyde-Zenker or Helly's fluid (the same as Zenker's fluid except that 40 per cent. formaldehyde is substituted for the glacial acetic acid). Potassium dichromate acetic acid (3 per cent. potassium dichromate 95 ml., glacial acetic acid 5 ml.). Mercuric chloride acetic acid (saturated aqueous mercuric chloride 95 ml., glacial acetic acid 5 ml.). Bouin's fluid. 'Susa'.

(ii) The single substances were acetaldehyde, 13 per cent. (to give a molecular concentration roughly equivalent to that in formaldehyde-saline); benzaldehyde (pure); ethyl alcohol, 80 per cent.; potassium dichromate, 3 per cent.; mercuric chloride, saturated aqueous solution; acetic acid, 10 per cent.; osmium tetroxide, 2 per cent.; chromic acid, 1 per cent. The solvent or diluent was distilled water in all cases.

(*i*) The stains used were: Gram's stain; periodic acid/Schiff; and acid fuchsin/aniline blue/orange G. Details of composition and technique were as given in our last paper (1952).

(*j*) Appropriate controls were used throughout. If, say, Gram's stain was being used on a section of Susa-fixed rabbit pituitary, a section of formaldehyde-fixed rabbit pituitary was stained side by side with it. When a number of pieces were embedded together in one block, one of them was always formaldehyde-fixed and was regarded as a control for the Gram staining. When a section from such a block was stained by Gram's method, the next sections in the series were stained by the periodic acid/Schiff method, or by acid fuchsin/aniline blue/orange G, or both, to act as a control for the presence of basiphil cells.

(*k*) On suspicion that results were influenced by the thickness of the sections, sections were cut from a formaldehyde-fixed rabbit gland at 5, 10, 15, and 25 μ, and stained by Gram's method as usual.

Results

First, it should be made clear that our study has been concentrated on the granules of the basiphils. Other structures (such as nuclei, collagen, and elastin) may also be Gram-positive. We have noticed this in our material, but the ensuing reports refer only to the basiphil granules.

The results may be briefly summarized by saying that the Gram reaction was positive only in preparations treated with formaldehyde-saline, formaldehyde-Zenker, Zenker's fluid, and Zenker stock. (See table 1.) This was constant when the glands were initially fixed in these fluids. A positive result was

Table 1. *Results of Gram reaction in basiphil granules of pituitary, after various fixatives*

Fixatives	*Gram reaction*
(i) *Mixtures*	
10 % formaldehyde-saline	+
Zenker's fluid	+
Zenker stock	+
formaldehyde-Zenker	+
potassium dichromate acetic acid	—
mercuric chloride acetic acid	—
Bouin's fluid	—
'Susa'	—
(ii) *Single substances*	
13 % acetaldehyde	—
benzaldehyde	—
3 % potassium dichromate	—
sat. aqueous mercuric chloride	—
sat. aqueous picric acid	—
10 % acetic acid	—
2 % osmium tetroxide	—
1 % chromic acid	—

usual, but not invariable, when frozen sections of unfixed glands, or sections of frozen-dried glands, were left in formaldehyde-saline for at least 12 hours. Frozen sections of unfixed glands, and sections of frozen-dried glands stained without further treatment, were always Gram-negative.

Fig. 1 (plate). A, low-power view of a section of four pieces of a human pituitary, separately fixed but embedded, cut, and stained together. Gram's method (no counterstain). The letters indicate the fixatives used (*f* = formaldehyde-saline, *z* = Zenker's fluid, *b* = Bouin's fluid, *a* = 80 per cent. alcohol). The small rectangles in the *f* and *a* sections indicate the positions of B and C.

B shows the typical appearance of the basiphil cells stained by Gram's method after fixation in formaldehyde-saline. Only basiphil granules are stained. (From the place shown in A, *f*; for comparison with C.)

C shows the appearances observed with staining by Gram's method after fixation in 80 per cent. alcohol. Nuclei and colloid are stained, but no granules. (From the place shown in A, *a*; for comparison with B.)

From this we conclude that the fixation, and not subsequent processes, is responsible for the results of staining by Gram's method.

Negative results were obtained with material fixed in potassium dichromate/acetic acid, mercuric chloride/acetic acid, and all the other mixtures and single substances mentioned above.

The staphylococci were Gram-positive in all cases. The conclusion here is that the Gram reaction in these organisms is different from that seen in the pituitary, which is perhaps not surprising.

Differences in the thickness of sections were not found to affect the Gram reaction. This test was prompted by the negative Gram reaction in the unfixed frozen sections: we suspected at first that this might have been due to the greater thickness of the frozen sections compared with the paraffin ones.

The results with the controls—see 'Methods (*j*)'—were not altogether clear-cut. When a section of a formaldehyde-fixed gland was used as a control for, say, a section of a Zenker-fixed gland, then the control was always Gram-positive. When a formaldehyde-fixed piece was embedded as a control along with pieces fixed in other reagents, then the formaldehyde-fixed piece was always Gram-positive. But, when neighbouring sections in the series cut from such blocks were stained with periodic acid/Schiff or acid fuchsin/aniline blue/orange G, as a control for the presence of basiphils, the results in the pieces fixed in single substances were so unsatisfactory that we could not be sure whether or not basiphil cells were in fact present. This doubt applied particularly to the small pieces embedded together. Formaldehyde-saline always gave positive results with periodic acid/Schiff.

We consider, however, that we are justified in reporting the negative results given above, for the following reasons. First, the results were negative in whole glands, and in glands that were halved or quartered. Secondly, the reports are based on two or more observations (except for mercuric chloride/acetic acid). Thirdly, the human glands were from elderly subjects, and basiphil cells were very numerous in them.

The cytoplasmic appearances in preparations reported as Gram-negative were not identical in all cases. In formaldehyde-fixed specimens the Gram-negative cells were invariably of a clear yellow colour. In contrast to this, in preparations fixed by potassium dichromate/acetic acid, and to some extent in those fixed by chromic acid, many cells showed a brownish or purplish coloration which at first sight suggested a rather poor Gram-positive result. The coloration however was not only much weaker than the true Gram stain, but also it was diffuse and not restricted to the granules; therefore we cannot regard it as a typical positive result.

Discussion

It seems to us that the salient findings are these: that, on the one hand, positive results are got with formaldehyde-saline, with formaldehyde-Zenker, Zenker's fluid, and Zenker stock, while, on the other hand, negative results follow fixation in acetaldehyde and benzaldehyde, in potassium dichromate

alone and potassium dichromate/acetic acid, in chromic acid, in mercuric chloride alone and mercuric chloride-acetic acid, and in Bouin's fluid.

The positive Gram reaction after formaldehyde, contrasted with the negative results after acetaldehyde and benzaldehyde, may be regarded as yet another instance of a property of formaldehyde not shared by some other aldehydes.

Examination of the other results seems to show that Gram staining in the basiphil granules of the pituitary depends upon fixation being done in either formaldehyde alone or mercuric chloride and potassium dichromate together. This conclusion in turn depends upon the assumption (based on appearances) that the Gram-positive results obtained by these two methods are in fact identical. We feel justified in making this working assumption, keeping in mind that it may later be proved erroneous.

The next problem appears to be to find a reaction or reactions on cell components, for example proteins, that is likely to be shared by formaldehyde and mercuric chloride plus potassium dichromate (see French and Edsall, 1945). We hesitate to do more than state this problem; the more so because formaldehyde and potassium dichromate are not protein precipitants, while mercuric chloride is (Baker, 1945). We wonder if perhaps in this combination the potassium dichromate effect predominates over or prevents the effect of the mercuric chloride; if that occurred, the mercuric chloride might act on the potassium dichromate-fixed material in such a way as to produce a Gram-positive result, which is not given by either substance alone.

A similar problem is presented by the constantly positive results with formaldehyde, in contrast to the negative results with Bouin's fluid (which of course contains formaldehyde).

The effect of formaldehyde on unfixed frozen sections and frozen-dried sections also interests us. The Gram stain was negative unless the formaldehyde was allowed to act for a number of hours, although the reagent must have penetrated quickly to all parts of these very 'thin slices'. This suggests a chemical reaction. Crawford and Barer (1951) found that morphological changes in cells continue for many hours during fixation by formaldehyde.

The results obtained in our work suggest that authors who describe staining-methods should be careful to specify exactly the fixatives they use.

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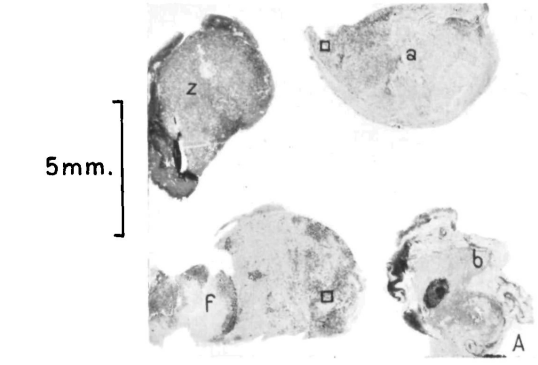

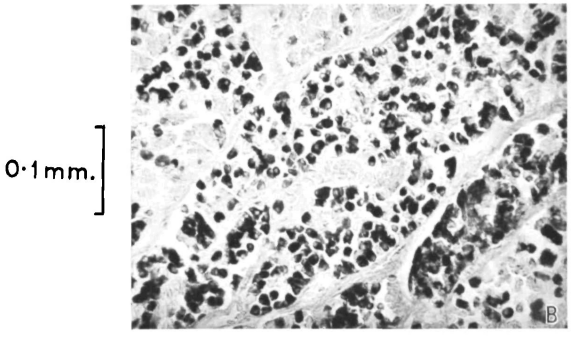

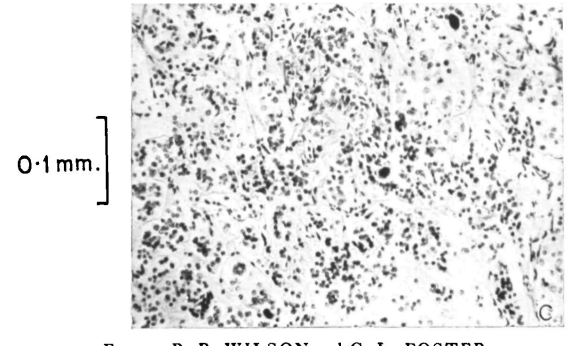

FIG. 1—R. R. WILSON and C. L. FOSTER