An Examination of Baker's Acid Haematein Test for Phospholipines

BY

A. J. CAIN

(From the Department of Zoology and Comparative Anatomy, Oxford)

CONTENTS

PAGE

INTRODUCTION 467
MATERIALS AND METHODS 467
RESULTS AND DISCUSSION 468
Standard results obtained with the acid haematein and pyridine extraction tests . 468
The stages of the tests 469
Investigation of the tests 470
(*a*) Role of the calcium ions in fixation and mordanting 470
(*b*) Evidence for a chromium compound in the tissues 472
(*c*) Completeness of extraction by pyridine 473
(*d*) Substances staining after pyridine extraction but not after acid haematein . 473
(*e*) Specificity and the period of chroming 474
(*f*) The blue and brown colorations 475
SUMMARY 477
REFERENCES 478

INTRODUCTION

THE only histochemical test for lipines that was regarded as specific by Lison (1935, 1936) is that of Smith-Dietrich. Baker (1946) has criticized it on the grounds that it is inadequately specified, not very sensitive, and gives a negative reaction with pure lecithin. He proposes his acid haematein test, based on the Smith-Dietrich test but with a pyridine extraction control, as being free from these defects. He has since shown (1947) that the test is for phospholipines only.

It is important to try to assess the value of this test. So many 'histochemical' tests have been shown to be unspecific, and the results obtained by them thereby invalidated, that caution is necessary in admitting the specificity of a new one. It would be better to have no tests rather than unreliable ones.

This paper presents the results of an examination of Baker's test, and gives reasons for believing that, provided only a definitely positive result is considered, it is specific, though there are certain possible sources of invalidity, as Baker has pointed out.

MATERIALS AND METHODS

Transverse sections of the rhynchobdellid leech *Glossiphonia complanata* (L.) were found to give a good variety of tissues, with plenty of lipine in the

fat-cells. Liver, kidney, testis (for interstitial cells) and small intestine of the mouse, and adrenals of the rat were also used. In addition, tests were made on paper impregnated with various pure substances and mixtures. Some of these were repeats of Baker's (1946) tests; all were carried out in accordance with the instructions given by him, except that in some cases the paper was soaked in the melted substance or mixture, and was therefore very thickly coated.

The method employed was to determine what results were given by the acid haematein and pyridine extraction tests when performed in exact accordance with Baker's instructions, and then study the effect of varying the chemical and physical components of the tests. For controls, sections and papers were coloured with sudan black.

Diphenyl-carbazide was used for detecting chromium.

Results and Discussion

Standard Results obtained with the Acid Haematein and Pyridine Extraction Tests

For convenience, these tests will be referred to as the AH and PE tests respectively. A blue, blue-black, or black coloration given by AH but not by PE indicates phospholipine.

The first point that is noticed on examining an AH section of *Glossiphonia* is that the nuclei, except for the plasmosomes, are nearly colourless. The mitochondria of the nephridial cells, the Golgi apparatus of the stomach epithelial cells, and usually the whole of the cytoplasm of the intestinal cells are blue, as are the cytoplasm of the fat-cells, and granules in the muscle-cells and in certain corpuscles in the coelomic spaces. The epidermis (except for the mucous cells) and the dermis are blue-black or black. Some of the dorsoventral and oblique muscle-fibre cortices may be blue also, but those of the longitudinal fibres vary from colourless through yellow to brown. The interfibrillar spaces in the longitudinal muscles are not usually coloured. The nerve-cords are pale blue, with dark blue in the nerve-cells. The cytoplasm of the salivary gland cells is very dark blue; the pigment cells remain pale brown, as in unstained sections. The cuticle is pale yellow.

Contrasts are seen immediately on examining a PE section. The nuclei are now full of small granules coloured a dark blue-grey. All the blue colorations mentioned above have disappeared completely or have been replaced by a faint blue-grey, except that the epidermis and dermis remain blue-black or black as before. Many of the interfibrillar spaces, particularly near the dermis, are now black or very dark brown. The cortices of the dorsoventral fibres are yellow or dark brown, those of the longitudinal ones, seen in cross-section, may be yellow, brown, black, brown with blue shadings, or deep blue. In one section they may all be yellow; in another, in each bundle there may be some yellow and some dark blue or dark brown. It is possible that the colour may vary with the degree of contraction. The pigment cells are now

full of dark blue granules smaller than those seen in AH and unstained formal-calcium preparations. The nerve-cords still contain some blue. The general tone of the cytoplasm is pale yellow, usually slightly darker than in AH preparations; the cuticle remains pale yellow.

It is evident, then, that in transverse sections of *Glossiphonia* we have examples of all the four classes of substances distinguished by Baker, thus:

(A) AH positive, PE positive. Epidermal cells, plasmosomes.

(B) AH negative, PE positive. Chromatin, pigment granules, interfibrillar matrix.

(C) AH positive, PE negative. PHOSPHOLIPINES. Cytoplasm of fat-cells, salivary gland cells, and intestinal cells, mitochondria of nephridial cells, Golgi apparatus of stomach epithelial cells.

(D) AH negative, PE negative. Fat droplets, cuticle, cytoplasm of nephridial and other cells; and cortex of muscle cells frequently.

The Stages of the Tests

The two tests may be summarized as follows:

AH. (i) Fix in formal-calcium, a non-precipitant fixative specially designed to prevent the loss of lipines (Baker, 1944).

(ii) Soak in potassium dichromate-calcium chloride solution at room temperature and then at 60° C., to make the phospholipines insoluble and mordant them. Wash well in water.

(iii) Cut frozen sections at 10 μ. (Embed in gelatine if necessary.)

(iv) Mordant again in the same fluid at 60° C. for an hour only, as the chromium compound left in the tissues to act as mordant may be washed out of small particles of lipine (Baker, 1946).

(v) Stain in acid haematein for 5 hours at 37° C.

(vi) Differentiate in borax-ferricyanide for 18 hours at 37° C.

(vii) Mount in Farrants's medium.

PE. (i) Fix in weak Bouin to render the phospholipines easily extractable by solvents and increase the power of proteins to give a positive reaction (Baker, 1946).

(ii) Wash in alcohol to remove picric acid.

(iii) Soak in pyridine, first at room temperature then at 60° C., to remove phospholipines (and all other lipoids).

(iv) Wash in water, then go to stage (ii) of the AH test and proceed as for it.

On consideration of these summaries the following questions arise:

(*a*) A calcium-haematein lake is easily produced by adding calcium chloride solution to some acid haematein, alkalinizing, and filtering off and washing the precipitate. A purplish-blue solid is obtained, which turns rusty

red on prolonged exposure to the air. It is insoluble in borax-ferricyanide but dissolves readily in acid haematein. Do the calcium ions in the fixing and chroming solutions assist in mordanting?

(*b*) What evidence is there that a chromium compound is left in the tissues?

(*c*) What is the degree of completeness of extraction by the PE method?

(*d*) Why do some substances stain after PE but not after AH? Obviously something more than a mere extraction has been performed.

(*e*) Does the specificity of the test depend on the period of chroming or on a reaction peculiar to phospholipines?

To which we may add after considering the results described above:

(*f*) How are both blue and brown colorations produced in the finished preparations?

Investigation of the Tests

(*a*) *Role of the Calcium Ions in Fixation and Mordanting.* Variations of the AH test as shown below were tried out to determine the effect of the omission of dichromate and calcium at different stages. All were done on *Glossiphonia* sections.

Variation	*Result*
1. Fix in 4 per cent. formaldehyde, and omit chroming and differentiation.	Whole section dark reddish-brown. No blue.
2. Same as (1) but with differentiation.	General pale yellow-brown. No blue.
3. Fix in formal-calcium, omit dichromate from the chroming solution, omit differentiation.	General brown. Phospholipine in fat-cells slightly darker brown. No definite blue.
4. As (3) but with differentiation.	Pale brownish-yellow throughout. No blue.
5. Standard AH but with calcium chloride omitted from the chroming solution.	As standard AH results above, but blue reduced greatly. Positive results only in bases of intestinal cells and cytoplasm of a few fat-cells.

The last variation was tried on other tissues also. With rat adrenal, considerably less blue was seen than in standard preparations. With mouse small intestine there appeared to be no reduction in blue-staining, and with mouse liver it was slight.

The conclusions from these results are that:

(i) dichromate is necessary for blue and almost all brown staining in the finished preparation,

(ii) it is necessary for the production of fast browns, and

(iii) calcium ions are necessary with dichromate for the maximum blue staining in some tissues.

Also, the behaviour of various lipoids in thick deposits on paper was investigated in the same way. The following results are representative:

Mixture	*Procedure*	*Colour at start*	*Colour after staining but before differentiation*	*Colour after differentiation*
Pinene saturated with cholesterol at 60° C.	Standard AH.	White.		General brown. Edges of some crystals medium to dark blue. Scattered dark blue granules.
	AH but omit calcium chloride from chroming solution.	White.	Very pale brown. A few small blue granules.	Pale greenish-brown, no blue.
	AH but omit dichromate.	White.	White.	White.
	AH but omit calcium chloride throughout.	White.	Very pale brown. A few small blue granules.	General pale greenish-brown. A few dark brown granules. A faint lilac tinge on some crystals.
	AH but omit dichromate, and calcium chloride from fixative.	White.	White.	White.

Exactly comparable results were obtained with an equimolecular mixture of cholesterol and oleic acid (BDH redistilled) and with oleic acid freed from all traces of phosphorus. In all cases, after the omission of dichromate no coloration (or only the faintest trace) was seen at any stage, but omission of calcium chloride had no effect. With these mixtures there is no loss of lipoid if calcium is omitted, as there is with phospholipine; so we may conclude that the calcium ions play no part in mordanting here, and it is very improbable that they do with phospholipines.

Examination of formaldehyde-fixed *Glossiphonia* sections with sudan black shows that a considerable amount of lipoid, including some phospholipine, is preserved without treatment with dichromate. Fixation is, of course, rather poor. It is not possible to say from examination of sections that less phospholipine is preserved after 4 per cent. formaldehyde than after formal-calcium, but it is seen from variation (5) on *Glossiphonia* and rat adrenals above that omission of calcium chloride from the chroming fluid does allow phospholipine to escape from some tissue, though not completely. It appears that the short fixation (6 hours) in formal-calcium is enough to fix proteins but not enough to affect the solubility of phospholipines if they are exposed to fluids at 60° C. Baker (1944) found in his studies of lipine solubilities that formaldehyde does make lipines less soluble in various solvents after 24 hours' fixation. One suspects that some lipine is lost even at room temperature if plain formaldehyde is used. If two pieces of lecithin paper prepared by Baker's method (1946) are fixed for 6 and 29 hours respectively in 4 per cent.

formaldehyde, left overnight in water at room temperature, and coloured with sudan black, it is seen that much more lecithin remains on the paper after the longer fixation. Little or none remains on paper fixed for 6 hours and soaked in plain dichromate solution at 60° for 24 or for 8 hours.

We may conclude that:

(i) under the conditions of the AH test neither formaldehyde nor calcium chloride, separately or in combination, will fix phospholipines, and

(ii) the calcium ions merely restrain the phospholipines from passing into solution. (It was for this purpose they were added to the fixing and chroming solutions by Baker.).

(*b*) *Evidence for a Chromium Compound in the Tissues.* It is seen from variations (i) and (iii) in section (*a*) above that a dark brown coloration can be produced in the tissues by acid haematein without the aid of dichromate, but it is reduced to a pale yellow-brown by differentiation; no blue is seen. Potassium dichromate is essential for the formation of the fast blue and brown colours; but a chromium lake is not producible by the method described above (part ii) for the calcium lake. Does the dichromate act as a mordant, or are the fast colours produced by the dye and oxidation products in the tissues?

Sections of *Glossiphonia* were prepared (*a*) as for AH and (*b*) with formal-calcium fixation but no chroming. Diphenyl-carbazide in 90 per cent. alcohol was used for the detection of chromium as anion. This substance gives colours varying from red to violet with several metallic cations and chromates, arsenates, molybdates, phosphates, and similar anions. (For an account and references, see *BDH Reagents for Delicate Analysis including Spot Tests*, 7th ed. 1939, BDH, London.) The unchromed sections were used as controls. Each section was washed in distilled water, laid on a slide, covered with one drop of a saturated solution of diphenyl-carbazide in 90 per cent. alcohol, and examined at once. (The reagent, if left exposed to the air, gradually goes pink by itself.)

Neither the controls nor the chromed sections gave any colour with the reagent applied alone. On acidifying with acetic acid, the controls still gave no colour, but a fine red was seen at once in the chromed sections. The coloured substance, unfortunately, is alcohol-soluble, and diffuses out rapidly, but on examining the section immediately after applying the reagent it was seen that the colour was most intense just where the deepest staining was seen in AH sections, that is, in the epidermis, intestine, fat-cells, and nerve-cord.

It appears then that a chromium compound, unstable in very acid solution, is to be found in the tissues, most plentifully where the staining is densest. The acid of the dye may have a slight differentiating effect on the mordant, perhaps.

As a confirmation, a paper test on lecithin and caseinogen, both of which give deep blue with AH, was carried out, unimpregnated paper being used as the control. All the papers were put through the AH test without section-

ing, the acid haematein being replaced by 2 per cent. acetic acid. After 24 hours' washing, both the lecithin and caseinogen papers gave strongly positive results and the controls were colourless. Washing for 2 more days reduced the colour in both cases but did not abolish it. (These tests might perhaps be criticized on the grounds that as it took 24 hours' washing before the controls were free from chromium compounds some chromium may have been trapped inside the papers under the layers of lecithin or caseinogen; the reduced reaction on further washing is not necessarily evidence that the chrome-lecithin or chrome-caseinogen compound is removed by washing.)

Pieces of bath-sponge stain deep brown after both AH and PE. After AH with no mordanting they stain deep brown but lose almost all their colour during differentiation. Mordanted pieces give a strongly positive result with diphenyl-carbazide and glacial acetic acid, but slowly. Also, if lecithin paper, unchromed, is stained in acid haematein, most of the lecithin is lost and there appears to be no staining. If acid haematein made up with 1 per cent. calcium chloride instead of distilled water is used, there is still no staining and little if any lecithin is lost.

Apparently a chromium compound is part of both blue- and brown-coloured substances. (The blue colour with dichromate and lipoids has, of course, been considered a chrome lake for very many years.) The above experiments do not rule out the possibility of the chromium combining with oxidation products of the lipoids rather than with the lipoids directly. Smith and Mair (1909) have shown that oleic acid will form an intermediate chrome compound in the course of oxidation to a dioxystearic acid.

(*c*) *Extraction by Pyridine.* The test of this is very simple. A *Glossiphonia* section was produced as for the PE test but was coloured with sudan black instead of acid haematein, and compared with an AH section similarly treated. The result shows that the extraction is very good. An exceedingly faint coloration is visible in places where phospholipine was very plentiful (notably in the fat-cells), and the rest is uncoloured. The contrast with the sudan-coloured AH section is most striking.

(*d*) *Substances staining after PE but not after AH.* From what has been said so far it is understandable that substances should be AH +ve and PE −ve, Ah +ve and PE +ve, or AH −ve and PE −ve, but it is a little difficult to see how the combination AH −ve and PE +ve can occur. The differences between the two tests are:

(i) the use of acetic and picric acids in the PE fixative;

(ii) the omission of calcium chloride from the PE fixative; and

(iii) the use of the strong base pyridine in the PE test for extracting lipoids.

This subject was not followed up in detail, but some points should be mentioned.

In *Glossiphonia* sections prepared as for PE but with the actual extraction omitted, the pigment-cell granules are much smaller and darker than in AH

sections, and nuclei, having been treated with a powerful protein precipitant, show dark granules of chromatin. It seems likely that in these cases the difference between AH and PE results is due to non-precipitant and precipitant fixation respectively. Baker has noted (1944) that chromatin in thick layers is coloured by AH. The colour of the pigment cells is probably entirely intrinsic and not due to staining.

The case of the interfibrillar matrix is more puzzling, but it should be noted that in exceptional instances it is coloured after AH. It is invariably coloured after PE but to a depth varying greatly in different individuals. In PE-fixed sections without extraction and chroming the pigment-cell granules are very dark (which confirms that their coloration is by concentration of their own pigment) but the rest of the section is a general yellow-brown, as in a PE section with only the extraction omitted. This seems to mean that the pyridine has some effect on the interfibrillar matrix, but the results vary in different individuals.

(*e*) *Specificity and the Period of Chroming.* Smith and Mair (1909) have investigated the action of dichromate solutions on lipoids, and their results are of great interest here. They found that many unsaturated lipoids would give a blue colour with haematoxylin after chroming for various periods, and even saturated ones could do so if mixed with cholesterol, which by itself was negative. The necessary period of chroming for unsaturated lipoids was shortened by adding cholesterol to them, in a few cases to 24 hours. With lecithin they obtained a negative result, almost certainly because, as Baker has suggested, there being no calcium ions present it went into solution. Kaufmann and Lehmann (1926), on examining the Smith-Dietrich test, considered it specific if a black colour only were taken as positive; blues, greys, and browns were given by various mixtures not containing lipines. Baker reduced the concentration of the stain considerably so that these weaker, non-specific results might be abolished. Two of Kaufmann and Lehmann's mixtures containing cholesterol or cholesteryl oleate but no lipines gave positive results, and Lison (1936) concludes that unless cholesterol or cholesterides can be excluded there remains a slight doubt. Smith and Mair found that a cholesterol-oleic acid mixture gave a clear blue after 24 hours' chroming at 65° C. with saturated potassium dichromate solution.

The following lipoids were therefore investigated, thick coatings on paper being used:

Oleic acid (phosphorus-free).
Cholesterol and oleic acid (BDH redistilled) in equimolecular proportions.
Cholesterol dissolved to saturation in pinene at 60° C.
Cholesterol dissolved to saturation in tributyrin at 60° C.

Details of the cholesterol-pinene results are given in part iii above. Cholesterol by itself is uncoloured by AH, but is solid at the temperatures of the test. All the above gave a few granules of pale or medium, and sometimes dark blue. Occasionally edges of thick masses of crystals were tinged bluish or

lilac. In the case of the oleic acid, staining produced a definite purple or purplish-blue which was almost completely removed during differentiation.

As was mentioned above, Kaufmann and Lehmann found that very many mixtures gave faint colours after the Smith-Dietrich test. Unfortunately, consideration of their results with nile blue (Cain, 1947) shows that some of their substances, including triolein or cholesterol in one case, were not pure. (It is worth noting here that their specimens of phrenosin and kerasin gave heavily positive results in mixtures containing no other lipine, and faint ones by themselves, with the Smith-Dietrich test. Baker has shown (1947) that a mixture of phrenosin and kerasin carefully freed from all traces of sphingomyelin (the most difficult of the phospholipines to remove) is negative to AH.) Consequently, their detailed results must be accepted with reserve, though supported in general by Smith and Mair's results and by those given above. At the same time, it must be remembered that they were the first to use extensive tests on pure substances and mixtures to assess the validity of histochemical methods for lipoids.

The weak colorations obtained with AH in mixtures on paper were never seen throughout the coating, and this was very thick, probably of the order of 100 or 200 μ, whereas the coloration of lecithin paper prepared according to Baker's instructions is present wherever there is lecithin on the paper and is quite a dark blue, although the total thickness is only about 10 μ (Baker, 1946). Very occasionally, as in a cholesterol-tributyrin preparation, there is a small cloud of granules of a medium blue but again only locally in a mass of the mixture far larger than could be expected in tissues. After study of the results it is concluded that a definitely positive result (dark blue or blue-black) with AH but not with PE does indicate phospholipines, but that with weak results (pale blues and greys) there is no certainty, unless, of course, a chemical analysis of extracts of the tissues shows that phospholipines are the only lipoids present. The intense coloration of mitochondria in many cells gives a good example of a positive result in very small bodies.

Prolonged chroming has been shown by Smith and Mair to produce blue colours with haematoxylin in many lipoids. (However, on repetition of one of their results, it was noted that chroming of the cholesterol-oleic acid mixture for several days gave with AH an increase in brown coloration but not in blue.) The specificity is therefore dependent on the period of chroming, which must never be increased. With AH, decreasing the period caused an increased response in the mitochrondria of mouse liver but a decreased one in those of mouse small intestine and kidney, and appeared to have no effect on the pale blues of the cholesterol-oleic acid mixture. It is therefore not recommended, as it may weaken the results obtained with some tissues and make them uncertain. A preliminary extraction with acetone to remove interfering lipoids was tried and gave fairly good but definitely fainter results and considerably increased the length of the test.

(*f*) *The Blue and Brown Colorations.* It was shown above (*b*) that a chromium compound is formed in the tissues in both blue- and brown-staining. If an

undifferentiated *Glossiphonia* section is examined, it is seen that what is blue in the differentiated section is blue there also and in much the same depth of colour. Differentiation, even for 88 hours, makes little difference to a *Glossiphonia* section as far as the blue stain is concerned. This is not true of the brown. In the undifferentiated section all that is not blue is dark brown except ground cytoplasm, which is nearly colourless or pale yellow. The effect of differentiation is to reduce the dark brown to pale yellow-brown or yellow. After 88 hours' differentiation, plasmosomes in the nuclei of intestinal cells have changed from black to brown. If, however, the progress of the AH test is observed with pure oleic acid (see (*e*) above) it is seen that a blue or purple colour is produced which is removed by differentiation. Consequently, it is important to keep to the prescribed period of differentiation. Shortening is not permissible; lengthening seems to have little effect on phospholipines in general, but might remove the colour from very small objects or weaken it to the point of uncertainty.

It will be noticed from Baker's results of paper tests that all the very dark blue-staining substances except legumin contain phosphorus. It is quite probable that such substances as fibrinogen, blood-albumen, mucin, nucleoprotein, nucleic acid, and perhaps trypsin might be impure. A faint positive result might almost be expected with blood-serum (rabbit) and blood-plasma (fowl). However, sodium glycerophosphate gave a negative result. But this substance dissolves in both formal-calcium and dichromate-calcium, so it is likely that it was lost from the paper at an early stage and the negative result is not significant. A negative result with a paper test means nothing unless it is shown that the substance was still present at the end of the test or that it would have been lost from tissues. A small quantity of this salt was dissolved in dichromate-calcium and the solution incubated at 60° C. for 18 hours as for chroming for AH. At the end of that time a crystalline precipitate had formed which was removed by filtration, when it was seen to be a very pale yellow, almost white. It was only slightly soluble in water, and a small portion tested with diphenyl-carbazide gave a faint red at once which was greatly increased on acidifying. (The first faint colour was almost certainly due to the retention of a little mother-liquor.) The rest was stained and differentiated as for the AH test. The resultant colour was black. This substance resembles the blue-stained substances in AH sections quite closely in its formation. One might suggest that rapid and intense blue staining is usually due to some combination between chromium and substituted phosphoric acids.

But this will not explain the case of legumin, and would lead one to suppose that at least some nucleoproteins would stain heavily, which is not so. Baker notes that all three dark-blue staining proteins (caseinogen, mucin, and legumin) are very acidic. Stearic, palmitic, and ricinoleic acids all gave a negative or exceedingly faint positive result, so the mere presence of an acidic group is not sufficient. Smith and Mair's work with oleic acid appears to show that chromium enters into combination at the double bond; prolonged

chroming abolishes the capacity to stain, a dioxy-compound being formed which contains no chromium.

The only safe conclusion appears to be that with the AH test, blue and brown colours are produced, both fast to 18 hours' differentiation with borax-ferricyanide at 37° C. The dichromate acts as a mordant in the blue staining and some at least of the brown, though it is not proved that in some cases it may not cause oxidation first. Under the conditions of the AH test mordanting takes place most readily with certain acidic compounds, especially some containing phosphorus. (This suggests that interference might come from bound phosphorylated sugars and other compounds in tissues if they are removed only by the action of pyridine.)

The production of different colours according to the substrate by the same dye *and mordant* under the same conditions has not, to my knowledge, been emphasized before, and seems worth investigation.

My thanks are due to Dr. J. R. Baker, who has supervised this work and discussed many of his unpublished results with me.

Summary

1. Baker's acid haematein test for phospholipines is specific provided that only a definite positively result is considered. Very pale blues and greys may be caused by other lipoids, which if present in very large masses may possibly show medium to dark blue granules but will not be coloured all through.

2. The mechanism of the test appears to be as follows:

(*a*) Phospholipine is not fixed by formal-calcium but is restrained from passing into solution by the calcium ions, which play no other part.

(*b*) Phospholipine combines readily with chromium from the mordanting fluid, and is then rendered insoluble and mordanted. Other substances, acidic and usually containing phosphorus, are mordanted as well.

(*c*) On staining, blue and brown colorations are formed; in both cases the dye attaches itself to the chromium in the various substrates.

(*d*) On differentiation, some browns and most blues, particularly those with phosphoric substrates, remain nearly fast, but most browns and the weak blues of certain lipoids (not phospholipines) are greatly reduced or removed entirely. The period of differentiation must not be shortened.

(*e*) Blue-staining lipoids (phospholipines) are distinguished from other blue-staining substances by an extraction with the lipoid solvent pyridine, after special fixation. The other substances, and any bound lipoid not removable with pyridine, remain.

3. Since the specificity of the test depends on the *relatively* greater affinity of phospholipines among lipoids for the mordant, the period of chroming must not be lengthened.

4. One reason why some substances are coloured after pyridine extraction but not after acid haematein is that in the former case they are precipitated and so concentrated; in the latter they are not. This is not a general explanation for the whole class of such substances.

REFERENCES

BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid. (in the press).
CAIN, A. J., 1947. Ibid. (in the press).
KAUFMANN, C., and LEHMANN, E., 1926. Virch. Arch., **261**, 623.
LISON, L., 1935. Bull. Histol. appl., **12**, 279.
—— 1936. *Histochimie animale, Méthodes et Problèmes.* Paris (Gauthier-Villars).
SMITH, J. L., and MAIR, W., 1909. J. Path. Bact., **13**, 14.