The Use of Nile Blue in the Examination of Lipoids

BY

A. J. CAIN

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

CONTENTS

INTRODUCTION

NILE blue was introduced by Lorrain Smith (1908) for distinguishing neutral fats (triglycerides) from fatty acids, the former being coloured red, the latter dark blue. Kaufmann and Lehmann (1926) applied the dye to a large number of mixtures of lipoids, and concluded that it was of no histochemical value at all. Lison (1935 *a* and *b*), after a profound study, concluded that the red coloration was characteristic of lipoids in general, and the blue was merely that of a basic dye and therefore totally unspecific. These conclusions supersede those in his book (1936).

This paper presents evidence that if a substance is known beforehand to be lipoid and colours red with nile blue, it consists of neutral lipoids (esters and/or hydrocarbons); if it colours blue, it may contain these, but acidic lipoids (fatty acids, phospholipines, and perhaps some others) are certainly present as well. The presence or absence of cholesterol cannot be established with nile blue.

The term 'nile blue' is used throughout to mean the commercial dye, which, as Lison has shown, is a mixture of the oxazine sulphate (true nile blue) and the oxazone (nile red).

MATERIALS AND METHODS

The tissues and cells examined were: adipose tissue and adrenal cortex of the rat, adipose tissue of the mouse, sebaceous glands in guinea-pig skin, and

fat-cells and stomach epithelium of the Rhynchobdellid leech *Glossiphonia complanata* (L.).

Formal-calcium fixation was always used, generally with postchroming as for Baker's acid haematein test for lipines (1946). Sections were cut on the freezing microtome, stained in a 1 per cent. aqueous solution of nile blue for 5 minutes, differentiated in 1 per cent. acetic acid for 30 seconds, washed, and mounted in Farrants's medium. In certain cases a 0·02 per cent. solution of nile blue was used.

Experiments were also carried out on various lipoids *in vitro*; these are described below.

Results and Discussion

I. *Observations on tissues*

It was observed that

(*a*) the fat droplets in the cells of rat and mouse adipose tissue always coloured red with 1 per cent. nile blue,

(*b*) the lipoid droplets in rat adrenal cortex cells (Harrison and Cain, 1947) and in the fat-cells of *Glossiphonia* might be red, blue, or some intermediate colour,

(*c*) the contents of guinea-pig sebaceous glands were always red, and

(*d*) the lipoid component of the Golgi body in stomach-epithelial cells of *Glossiphonia* stained deep blue, but went red after very prolonged differentiation (Cain 1947).

There is, then, a definite phenomenon to be investigated.

II. *Examination of Kaufmann and Lehmann's results*

(*a*) *General considerations.* Lison (1935*b*) concluded that the red substance that colours lipoids was not the free base of nile blue, as had been thought, but an oxidation product, an oxazone, which he named 'nile red'. Smith had shown this in his first paper, but his opinion had been disregarded, and he found it necessary to restate his views (1911) and insist that the double coloration was not a case of metachromasy as several authors had assumed.

The reasons why Smith's conclusion was not generally accepted were because (*a*) a red substance was readily extractable from aqueous solutions of nile blue by shaking with toluene or similar solvents, and (*b*) no such substance could be extracted from solid nile blue. Obviously the red substance was produced by hydrolysis, and must be the free base. Lison showed that the substance extractable from 1 per cent. or stronger solutions of nile blue was the oxazone, as Smith had stated, and that, being a very weak base, it formed salts which were insoluble in toluene and were hydrolysed instantly by water. The oxazone was insoluble in water, but soluble to some extent in fairly concentrated solutions of nile blue; and therefore could be extracted with toluene from strong aqueous solutions but not from the solid dye. It behaves like any other lipoid-colorant, such as sudan black or sudan IV, and

cannot be used to distinguish between the various classes of lipoid. Further, he showed that, as Smith had also stated, the free base (oxazine) is obtainable by extracting a concentrated solution of nile blue until all the oxazone is removed, alkalinizing, and extracting again. The solution obtained differs from an oxazone solution in the shade of red, and in being without fluorescence. The base is a strong one and readily combines with atmospheric carbon dioxide and moisture to give the blue carbonate, which, like the sulphate, is soluble in water but not in toluene and similar solvents. The intensity of coloration of the oxazine is far less than that of the oxazone, and it is the latter that colours lipoids in sections.

Lison also showed, for the first time, that whereas 1 per cent. or stronger solutions of nile blue are not hydrolysed, and the only red substance extractable from them is the oxazone, more dilute solutions are hydrolysed, and a mixture of oxazine and oxazone is obtained on extraction. With very dilute solutions almost pure oxazine is obtained, the oxazone being almost insoluble in them. (I have found a 0·02 per cent. solution satisfactory.) On standing, aqueous solutions of oxazine sulphate produce a certain amount of oxazone by oxidation.

He concluded that since the oxazine sulphate was merely a basic dye, and since the oxazone was merely a general lipoid-colorant, the use of the two together gave results of no more significance than would any other comparable combination, and that nile blue should not be used in histochemistry.

Nevertheless, such a combination might be used to distinguish between those lipoid mixtures that do and those that do not contain substances which will take up the blue colour. But Kaufmann and Lehmann (1926) had applied nile blue to a large number of lipoids and mixtures of lipoids and concluded that it was useless. To simulate tissue sections they enclosed the substances to be studied in pith, which was sectioned, stained, and mounted. Lison (1936) comments on their results that (*a*) every time a red colour was obtained, the mixture contained triolein, but many mixtures containing triolein were blue or uncoloured, and (*b*) 31 cases were observed in which mixtures containing free fatty acids (which according to Lorrain Smith should have stained blue) were uncoloured, and in 7 cases mixtures with no fatty acids at all were coloured blue.

The results they obtained with pure lipoids are of great interest. Oleic acid was blue, triolein red. Palmitic and stearic acids, tripalmitin, tristearin, cholesterol, cholesteryl oleate, lecithin (Merck and Kahlbaum), sphingomyelin, phrenosin, and kerasin were all uncoloured. Several of these substances were solid at room temperature, but might well have stained in solution. Others such as lecithin might be expected to stain even in the solid state.

A repetition of some of these results was therefore attempted with small portions smeared or melted on to coverslips. Since triolein was red, they must have used a fairly concentrated solution of nile blue, and a 1 per cent. solution was therefore employed. Tristearin and tripalmitin were

uncoloured, stearic and palmitic acids very faint blue, nearly white, cholesterol uncoloured. All these were solid. Cholesteryl oleate, which was greasy, was pink, and lecithin exceedingly dark blue. It is probable that Kaufmann and Lehmann obtained no result with lecithin because their method of impregnation of pith was not suitable or because it escaped from the sections. On warming tripalmitin with nile blue solution, it coloured red on melting. Triolein and tributyrin, being liquid, coloured red at room temperature.

From these experiments it is seen that Kaufmann and Lehmann's results require reinterpretation. In the first place, lecithin (and therefore perhaps other phospholipines) stains an exceedingly dark blue. In the second place, a negative result may mean merely that the mixture was solid.

(*b*) *Blue-staining mixtures containing triolein.* If the properties of mixtures of lipoids were the sum of the properties of their components, it should be possible to prophesy the result of staining with nile blue from data on pure substances. In view of the blue-staining properties of lecithin and the possibility that fatty acids other than oleic may stain in solution, the apparent exceptions among Kaufmann and Lehmann's results require re-examination. There are 46 cases in which mixtures are not coloured red, although triolein is included. Of these, 35 contained oleic acid (which is known to stain blue) and the result obtained was blue, or in one case *violetblau.* Of the rest, 5 contained lecithin, 1 phrenosin, and 1 kerasin, and all were coloured blue. It is interesting that phrenosin and kerasin seem to behave like lecithin. Baker (1946) noted that galactolipine varied in the response given to his test for lipines, and suggested that pure galactolipine did not respond. It is well known that the complete separation of galactolipine from sphingomyelin is very difficult, and sphingomyelin resembles lecithin and cephalin in containing phosphoric acid. Possibly the samples used by Kaufmann and Lehmann were not quite pure. The remainder of the exceptions are:

(A1) triolein and cholesterol—blue;
(A2) triolein and glycerol—part red, part blue;
(A3) triolein and cholesteryl oleate—*violettros*; and
(A4) triolein, cholesterol, and lecithin—part red, part uncoloured.

The second might have been expected, perhaps, as solid nile blue will dissolve in glycerol to give a blue solution. The third is at least reddish. But the first is wholly unexpected, and obviously requires confirmation.

It was tested in the following ways:

(i) A test-tube was half-filled with 1 per cent. nile blue solution, and some triolein run in. After shaking, the triolein layer was red. Cholesterol crystals were then dissolved in it until it was saturated. Even after prolonged shaking, it remained red, though with a change in tone towards cherry-red. The same result was obtained on repeating the experiment with xylene or tributyrin replacing triolein.

(ii) Nile blue dissolves in acetone, giving a blue solution with a distinct red tinge. Saturation of an acetone-nile blue solution with cholesterol produces no change in colour.

(iii) Addition of cholesterol to a solution of the oxazone in xylene has no effect on the colour.

(iv) Addition of cholesterol to a xylene solution of the free base (oxazine) changes the colour from a clear to a dirty, rather opaque red.

(v) Solid cholesterol, as shown above, does not stain with nile blue. Melted cholesterol colours bright orange-yellow with solid nile blue. As it crystallizes, the colour changes very abruptly to blue.

From these experiments it appears that cholesterol in solution is inert towards nile blue, and melted cholesterol acts only as a solvent. As was expected, there appeared to be no combination. One is forced to the conclusion that at least in this particular case either the cholesterol or the triolein used by Kaufmann and Lehmann was impure, or both were.

The fourth exception given above is also very interesting, as it seems to show that the presence of lecithin is not sufficient to ensure that a mixture will stain blue. In this case, a positive result was obtained with the Smith-Dietrich test at 60° C. and with Schultze's test for cholesterol, so apparently both lecithin and cholesterol were present in the mixture. The five mixtures that contained triolein and lecithin and stained blue were:

(B1) triolein, palmitic acid, and lecithin,
(B2) triolein, stearic acid, and lecithin,
(B3) triolein, stearic acid, cholesterol, and lecithin,
(B4) triolein, tristearin, cholesterol, and lecithin, and
(B5) triolein, cholesterol, glycerol, and lecithin,

the last being coloured blue or purple. The blue coloration of the fourth can only be attributed to lecithin or impurities, but in the case of the first three it is possible that the fatty acids may colour in solution in triolein.

On adding palmitic acid to a xylene solution of oxazone, there was no change of colour. On adding a few c.cm. of 1 per cent. nile blue solution and shaking, the colour of the xylene layer became a reddish purple. The interpretation of this result is that xylene, like triolein, will dissolve from 1 per cent. nile blue only the oxazone, which will not form a salt with a fatty acid. The fatty acid does form a blue salt with the oxazine, and this then dissolves in the xylene layer, turning its colour towards blue. Now Lison (1935*b*) showed that there is very little free oxazine in 1 per cent. nile blue, but a considerable amount in more dilute solutions. Shaking a very dilute solution of nile blue with a xylene solution of palmitic acid should therefore cause the xylene layer to be coloured blue, and this is found to be the case. Addition of a fatty acid to a xylene solution of free oxazine produces an intense blue immediately.

From these results it appears that the staining blue of the first three mixtures (B1, B2, and B3) is more likely to be due to lecithin than to the fatty acids, as Kaufmann and Lehmann used a strong solution of nile blue. The only conclusion possible with respect to mixture A4 above is that the results of the Smith-Dietrich test are not necessarily relevant to the results obtained with nile blue.

Since glycerol gives a blue solution with solid nile blue, it is possible that mixture B5 might be accounted for without invoking lecithin. B4 requires examination, as it contains tristearin.

> On dissolving tristearin in xylene or tributyrin solutions of either oxazine or oxazone, no change in colour was produced, nor was any blue imported into the xylene layer on shaking xylene solutions of triglycerides with strong or weak nile blue solutions. Melted triglycerides coloured red with 1 per cent. nile blue, and were almost uncoloured with 0·02 per cent. solutions.

It seems unlikely, then, that the blue colour of B4 can be attributed to anything but lecithin or impurities. The Smith-Dietrich test for this mixture gave a positive result, but so it did for mixture A4.

(*c*) *Red-coloured mixtures containing triolein.* The mixture (A4) of triolein, cholesterol, and lecithin which was partly red and partly uncoloured has already been mentioned. On a mixture of triolein and lecithin which coloured red Kaufmann and Lehmann comment *blaszt bald ab*, yet this mixture gave a positive result with the Smith-Dietrich test at 60° C. and therefore might have been expected to stain blue. It seems impossible to correlate the results of different tests upon the same mixture.

> On adding lecithin to a xylene layer over 1 per cent. nile blue and shaking, the xylene went from red to blue. On dissolving lecithin in a xylene solution of oxazone, there was no change in colour, but with an oxazine solution there was an immediate change to blue.

Apart from the two mixtures just mentioned, ten cases of red coloration of mixtures containing triolein, exclusive of those giving purple or other intermediate tones, were observed by Kaufmann and Lehmann. Of these, only one (C9 below) contained oleic acid. They were:

(C1) triolein and palmitic acid,
(C2) triolein and stearic acid,
(C3) triolein, palmitic acid, and cholesterol,
(C4) triolein, stearic acid, and cholesterol,
(C5) triolein and sphingomyelin,
(C6) triolein and glycerol (part red, part blue),
(C7) triolein, stearic acid, and glycerol,
(C8) triolein and albumen,
(C9) triolein, kerasin, and oleic acid,
(C10) triolein and cholesteryl stearate.

The first four are explicable if the mixtures were tested with a strong solution of nile blue. The tenth is to be expected if cholesteryl esters behave like triglycerides.

On dissolving cholesteryl acetate or stearate in solutions of oxazone or oxazine in xylene, no change in colour was produced. These esters do behave like triglycerides.

The rest, except C9, are not surprising, but as confirmatory experiments on sphingomyelin, phrenosin, and kerasin could not be done, no definite comments on them can be made. The only explanation for C9 is that there must have been very little oleic acid present.

No mixtures were coloured red that did not contain triolein.

(*d*) *Other mixtures, blue-stained or uncoloured.* Forty-two mixtures are stated to have been uncoloured, one is recorded as fugitive, and two (mentioned above) as occasionally uncoloured. Such mixtures as tripalmitin and palmitic acid, tripalmitin and stearic acid, palmitic acid and tristearin, palmitic acid and cholesterol, or tristearin and cholesterol one would expect to be uncoloured, as they are solid at room temperatures. Others are more surprising. Such mixtures as palmitic acid and lecithin, stearic acid and lecithin, tripalmitin and lecithin, and stearic acid with tristearin and lecithin, might be expected to stain at least superficially. Again, in the absence of confirmatory observations on pure sphingomyelin and galactolipines it is not possible to comment on some mixtures.

Considering only mixtures containing fatty acids, in twenty-three cases in which there was no coloration it is likely that the mixture was solid and could not take up the stain. In only one case, a mixture of oleic acid and kerasin, was there no coloration when it might reasonably have been expected. This same mixture gave a positive result with the Smith-Dietrich test at 60° C.

III. *Discussion*

It is seen from the above examination of Kaufmann and Lehmann's results, and the supplementary experiments, that in one case at least there is doubt as to the purity of the substances used by these workers, and that correlation of the results obtained by different tests on the same mixture is difficult. Most of the results, with some exceptions (one of which is directly contradicted by *in vitro* experiment) are consonant with the following propositions:

(*a*) Substances in the solid state (except greases) do not colour in any way with aqueous solutions of nile blue.

(*b*) Triglycerides, liquid or dissolved in hydrocarbons or other triglycerides, are coloured red, if pure, by the oxazone or, much less intensely, by the free base (oxazine).

(*c*) Fatty acids if liquid colour blue with solutions of oxazine or dilute nile blue solutions. With strong solutions of nile blue, only a slight change towards blue is seen except with oleic acid which colours fairly strongly.

(*d*) Lecithin (and perhaps all phospholipines) stains deep blue when solid It has no effect on oxazone solutions, turns oxazine solutions blue, and colours blue with weak or strong aqueous solutions of nile blue.

(*e*) Cholesterol in solution (in fat-solvents) is inert towards nile blue, and the solution will not dissolve nile blue sulphate.

In connexion with the last proposition it is interesting to note that acetone and ethyl alcohol will dissolve the sulphate, but terpineol behaves like cholesterol and its esters, triglycerides, xylene, toluene, benzene, carbon disulphide, and pinene.

The principles underlying these propositions are that neutral lipoids will dissolve out of aqueous solutions of nile blue only the oxazone and free base (the latter not being present in appreciable quantities in 1 per cent. solution) but acidic lipoids will dissolve the oxazone and combine with the free base to give blue compounds which are soluble in lipoids and may mask the red coloration due to the oxazone. Alcohols act as solvents, the water-soluble ones dissolving oxazine, oxazine sulphate, and oxazone, the other dissolving only the oxazine and oxazone.

It will be noted that only lecithin and oleic acid will colour blue to any great extent with 1 per cent. solutions of nile blue. Oleic acid is of the order of strength of stearic and palmitic acids, but these require the free base in quantity to colour well. In view of the intersolubility of lipoids, the great difficulty of preparing them pure except by synthesis, and the natural origin of most oleic acid, it is possible that the samples used contained a certain amount of lipine and so could colour blue even with 1 per cent. solutions. Alternatively, it is just possible that a small amount of oxazine sulphate can dissolve in oleic acid and so colour it. Certainly, the intensity of staining of lecithin appears much greater than that of oleic acid, which makes it probable that lipoids staining blue in tissue sections with 1 per cent. nile blue contain lipine. However, this cannot be used yet to give histochemical conclusions.

To make full use of nile blue one must employ both the oxazone and the oxazine. The former is the intense colorant for lipoids, the latter the reagent for fatty acids. Further, the lipoids under investigation must be liquid. The following procedure is suggested:

(i) Fix in formal-calcium, with or without postchroming.

(ii) Cut sections on the freezing microtome.

(iii) Colour one section in sudan black, to define the lipoids present.

(iv) Stain one section in 1 per cent. nile blue at 60° C. or at 37° C., for 5 minutes, wash quickly in water at the same temperature, and differentiate in 1 per cent. acetic acid for 30 seconds, still at the same temperature. (It is necessary to work at the same temperature throughout, otherwise some lipoid may entrap a small amount of stain in crystallizing, and this will not be removed by washing and differentiating.)

(v) Mount in Farrants's or some similar medium. This section will show what lipoids will colour blue with 1 per cent. nile blue.

(vi) Repeat with another section and immediately after staining restain in a 0·02 per cent. solution of nile blue at the same temperature, then wash, differentiate, and mount as before. Comparison of the two nile blue sections should then indicate where in the tissues fatty acid is to be found, if there is any other than oleic acid present.

If there is no observable difference between the two sections, then the first may be dispensed with, as what will stain blue with 1 per cent. nile blue will stain with the 0·02 per cent. solution.

There are certain points to be noted when interpreting results. In the first place, only purely lipoid inclusions can be considered. Nile blue sulphate is a basic dye, as Lison has reminded us, and is not in the least specific for lipoids. It will stain most elements of a tissue, and it is not possible to distinguish between blue-stained lipoids and other substances by prolonged differentiation. It is true that lecithin is still deep blue after 24 hours' differentiation, which may be useful at times as an indication, but some acidic proteins are also still stained after this period. There is still a faint blue in nuclei, for example. It is not permissible to prepare sections for Baker's acid haematein and pyridine extraction tests, stain them in nile blue, and compare them. The pyridine, perhaps because it is a strong base, has a definite depressant effect on staining by nile blue sulphate in regions where no lipoid can be shown by sudan black. The only safe method for determining whether a body is purely lipoid is to colour pyridine-extracted sections with sudan black and see if it has been completely removed. If not, no conclusion can be drawn from results obtained with nile blue, unless it can be shown that the residue after extraction with some other lipoid-solvent will not stain blue, or will do so only with markedly reduced intensity. Also, if a body will colour only red with nile blue, it does not necessarily mean that acidic lipoids are completely absent. But, of course, if a blue-stained body can be turned red by prolonged differentiation, that is proof that lipoid is present. This method has been used (Cain, 1947) to apply the oxazone in an aqueous medium to a structure in which the use of the enormously powerful and opaque sudan black showed no details at all. The oxazone did not render the body opaque, and so revealed some details of its construction. Finally, such lipoid inclusions as cholesterol crystals will not liquefy at 60° C. and cannot be examined with nile blue.

My thanks are due to Dr. J. R. Baker, who has supervised the work set out in this paper.

Summary

Nile blue, introduced by Lorrain Smith for distinguishing between neutral fats (triglycerides) and fatty acids, was considered by Lison to be of no histochemical value except that the red coloration was specific for lipoids in

general. This conclusion was based principally on Lison's own researches, and in part on those of Kaufmann and Lehmann.

Reinvestigation has shown that some of Kaufmann and Lehmann's results are of doubtful value and all require careful interpretation, but in general these and other results lead to the conclusion that nile blue can be used to distinguish neutral lipoids (esters and hydrocarbons) from acidic lipoids (phospholipines and acids). Cholesterol is not detected.

A method for using both the oxazine and oxazone of nile blue is described, which has greater sensitivity to acidic lipoids.

REFERENCES

BAKER, J. R., 1946. Quart. J. micr. Sci., **87**, 1.
CAIN, A. J., 1947. Quart. J. micr. Sci., **88**, 151.
HARRISON, R. G., and CAIN, A. J., 1947. J. Anat. (in the press).
KAUFMANN, C., and LEHMANN, E., 1926. Virchows Arch., 261; 623.
LISON, L., 1935*a*. Arch. Biol., **46**, 599.
—— 1935*b*. Bull. Histol. appliquée, **12**, 279.
—— 1936. *Histochimie animale, Méthodes et Problèmes*. Paris (Gauthier-Villars).
SMITH, J. LORRAIN, 1908. J. Path. Bact., **12**, 1.
—— 1911. J. Path. Bact., **15**, 53.