A Critique of the Plasmal Reaction, with Remarks on Recently Proposed Techniques

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INTRODUCTION

THERE has been much disagreement between histochemists and biochemists over the chemical basis of the plasmal reaction and the techniques to be employed in demonstrating it. The purpose of this paper is to provide a critique of the methods that have been proposed.

INVESTIGATIONS BY THE FEULGEN SCHOOL

The occurrence in cytoplasm of substances giving a positive result with Schiff's reagent ('fuchsine-sulphurous acid') was first announced by Feulgen and Rossenbeck (1924, p. 230) in a footnote to a paper on the nucleal reaction. In the same year Feulgen and Voit showed that the substances responsible, which were adsorbed on to certain elastic-tissue elements so strongly as to resist paraffin embedding, were aldehydes, as the reaction could be abolished rapidly by phenylhydrazine and by sodium bisulphite. Similar substances found in frozen sections of fresh tissue were demonstrated to be lipoid by their solubility in alcohol; and Voss, who studied the resistant substances (1927, 1928, 1931*a* and *b*), showed (1927, p. 586) that the longer the tissues were in alcohol, the weaker the reaction became.

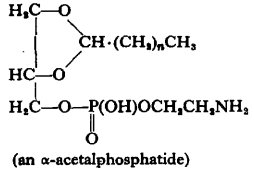
Feulgen and Voit tried the effect of mercuric chloride on tissues and discovered that the reaction became intensified, or appeared in tissues previously negative; but the same final result could be obtained by the prolonged action of Schiff's reagent, even after treatment with phenylhydrazine, the phenylhydrazone being decomposed by the acidity of the reagent. In tissues negative without the action of mercuric chloride, a preliminary treatment with alcohol

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

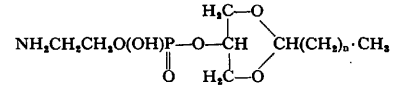
abolished the positive reaction after mercuric chloride. Therefore there was a lipoidal precursor which gave rise to the aldehydes, rapidly under the influence of mercuric chloride, slowly in acid media. They named the aldehydes 'plasmal' to mark the distinction in topography between this and the 'nucleal' reaction; the precursor they named 'plasmalogen'. Extracts of tissues were made, the reaction was obtained *in vitro*, and the thiosemicarbazone silver salt of the aldehydes was obtained in the pure state.

An important series of papers followed, on the occurrence of plasmalogen and plasmal in the body fluids, on their ingestion with food (Voit, 1925; Stepp, Feulgen, and Voit, 1927; Feulgen, Imhäuser, and Westhues, 1928; Imhäuser, 1928), and on their quantitative estimation in body fluids (Feulgen and Imhäuser, 1927; Feulgen and Grünberg, 1939), but these are not specially significant from the point of view of histochemistry. But Imhäuser in 1927 carried out a survey of the occurrence of plasmalogen and plasmal in tissues, and concluded that they were very widespread indeed, in fact that all animal cells contained them, and therefore they must be of fundamental importance in the physiology of the cell. The literature up to 1929 was reviewed by Grevenstuk (1929).

The chemistry of plasmal and plasmalogen was further investigated with great success. Palmital and stearal were investigated by Feulgen and Behrens (1928), who with Imhäuser showed (1929) that plasmal could be isolated and identified by comparison as palmital with an admixture of some stearal. But one other aldehyde at least was present which could easily be detected by its strong smell. This aldehyde could not be isolated, and they believed it to be unsaturated. They had discovered that plasmalogen was always found in the phospholipine fraction on extraction, but it was not itself a phospholipine, or formed from one in the course of extraction, since the content varied according to the source of the extract. Egg yolk, for example, has a very high content of phospholipine and only traces of plasmalogen, whereas the phospholipines of muscle or brain might contain 10 per cent. plasmalogen or more. Separation of the plasmalogen from the phospholipines could be accomplished only by saponifying the latter in alkaline solution; in acid solution, of course, plasmal was rapidly produced. Behrens (1930) prepared plasmal by an improved method, and Feulgen and Behrens (1938) obtained palmitic and stearic acids from it by oxidation. The work was surveyed by Feulgen and Bersin (1939) who showed in addition that plasmalogen could be isolated, though with great difficulty, and that it had the structure of an acetalphosphatide (α or β), that is,



(an α-acetalphosphatide)

or 

(a β-acetalphosphatide)

where the fatty acid radicle is palmityl or stearyl (or the unidentified unsaturated acid). It was observed that the base found was always cholamine. They suggested that choline might replace it, but possibly the acetalphosphatides containing choline were decomposed during the alkaline saponification of the other phospholipines, without which, unfortunately, isolation could not be accomplished. They pointed out also that almost certainly the reason why the acetalphosphatides remained unknown for so long, while the other phospholipines were carefully investigated, was that in the analysis of the phospholipines acid media were invariably used at some stage, and these would destroy the acetal linkages, leaving merely the aldehydes and glycerophosphoric esters. Finally Bersin *et al.* (1941) achieved the synthesis of acetalphosphatide. This appears, unfortunately, to be the last paper published by Feulgen's school; if so, it fittingly terminates their excellent researches.

In the investigation of the occurrence of plasmal, Feulgen and Voit (1924) and Imhäuser (1927) used fresh, unfixed sections, keeping some as controls and treating others with 1 per cent. mercuric chloride. They stuck sections on to slides with albumen, let them dry, and 'fixed' them by quick passage through a flame. They laid very great stress on the necessity for a control section untreated with mercuric chloride—a point that was to be almost completely neglected by other workers on plasmal until Gérard (1935) showed that formaldehyde could be used as a fixative provided it was carefully washed out. This was confirmed by Lison (1936*b*) and the practice was followed by Verne (1947*a* and *b*) but not Verne and Verne-Soubiran (1942). Voss (1927–31*b*) and Verne in earlier and some later papers had used a fixative containing mercuric chloride in order to obtain good fixation, thereby depriving themselves of the control section.

Investigations by Verne

The results obtained by the histochemists differ somewhat from those obtained by the Feulgen school, and are not wholly self-consistent. The principal worker on the plasmal reaction has been Verne, who has made a very extended survey of many tissues in different animals, both normal and in various induced pathological states, and has come to extremely important conclusions. He showed (1928*a*) that the plasmal reaction is by no means as widespread as Feulgen and Voit, Imhäuser, and others had thought. Many cells and tissues are completely negative, others (e.g. intestinal epithelium, pancreas, muscle) give only a feeble reaction, but the adrenal, myelin, certain parts of the kidney, and certain cells in the lung are intensely positive. The medulla of the adrenal is weakly positive, the z. glomerulosa, outer part of the z. fasciculata, and inner part of the z. reticularis are strongly positive.

The 'spongiocytes' (fat-laden cells) of the z. fasciculata are very weak or negative, as is adipose tissue. He noted that there was a relation between the reaction and lipoids, but no complete parallel. The reaction with the myelin sheath was so intense that he proposed it as a method for colouring the sheath (1928*b*, *d*) after fixing in a mercuric chloride or platinum chloride fixative and cutting frozen sections. On further investigation of plasmal (1928*c*), in addition to noting that sodium bisulphite and phenylhydrazine block the reaction, and that, as mentioned above, platinum chloride can also be used to provoke its appearance, he distinguished three types of lipoid inclusion: (*a*) positive with sudan III and negative with plasmal, (*b*) positive with both, and (*c*) negative with sudan III but positive with plasmal—the lipoid nature of the last being demonstrated by their solubilities. He made the important observation that by the use of oxidizers, such as potassium permanganate, chromic oxide, or hydrogen peroxide, plasmal-positive bodies could be changed to negative, and plasmal-negative ones to positive, and concluded (p. 268) that such bodies were formed from either neutral fats or phospholipines by oxidation of a suitable hydroxyl group to an aldehyde. He assumes, therefore, that since he has been able to produce positive results by the action of oxidizers on lipoids, those lipoids which already give positive results must already be oxidized in the body and in the same way.

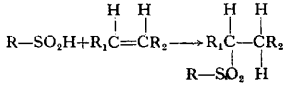
Verne summarized and extended his results in his next paper (1929*a*) in which he makes the interesting remark (p. 248) that fixation should be for at least 6 hours. He points out that, as Feulgen and his collaborators had stressed, a positive result is indicated by a red colour which is much more bluish than that given by Schiff's reagent which has been allowed to become recoloured through the loss of sulphur dioxide. He notes that sebaceous glands are negative, that the interstitial cells of the testis are positive, and that the adrenal is the only other endocrine gland studied which was positive. His results are based on studies of man, dog, cat, cow, calf, sheep, chick, common frog, tench, and eel. In experiments with oxidizing agents he found that adipose tissue can be made positive with plasmal, and that in general, as a lipoid body becomes positive through oxidation the intensity of colouring which it will assume on treatment with sudan III progressively decreases and becomes negative. Sections of adrenal left in water with a trace of mercuric chloride showed a positive result in the z. fasciculata; the same effect was produced in a quarter of an hour by 0·5 per cent. permanganate, or after several hours with 1 per cent. chromium trioxide. He mentions certain renal tubes and pulmonary cells and the adrenal medulla as negative to sudan III and positive to plasmal, and remarks that the pulmonary cells will withstand paraffin embedding. Researches on pure substances showed that pure glycerides and fatty acids (he does not say which), and cholesterol are always negative. Oleic acid and triolein are negative when pure, but become positive after exposure to the air. Unsaturation appears to be necessary, except that saturated aldehydes will give the reaction; unsaturated aldehydes are not present since they cannot be regenerated from the sodium bisulphite com-

pound by dilute acid, whereas the reaction can be made to reappear in tissues after treatment with sodium bisulphite. Consequently he revises his previous conclusion that the aldehydes concerned are formed from alcohols, and now considers that they are formed by oxidation at double bonds, which would explain why it is only the saturated aldehydes that are in question. As aldehydes are notoriously reactive, they are probably in some sort of labile combination in the tissues (those, that is, that have already been produced *in vivo*). The combination is broken by the action of the mercuric chloride or platinum chloride in the fixative. Free aldehydes, he states, have never been found in the tissues. He tried the action of mercuric chloride on various lipoids *in vitro* but could obtain no result.

Verne has not departed from these conclusions, but has strengthened his evidence for them in a long series of papers: on the adrenals (1929*b*), on the action of carotenoids in protecting lipoids from oxidation (1936*a*, *c*), on the acceleration of oxidation by glutathione and colchicine (Verne and Verne-Soubiran, 1939), and especially on the lipoids of the kidney (1937*a*, *c*, 1940).

Investigations by Lison and Gérard

However, certain objections to the specificity of the plasmal reaction had been raised. Lison (1932), after a very careful study of Schiff's reagent, showed that it was by no means specific for aldehydes, although aldehydes were among the most familiar compounds that would give a positive result. He reviewed the work of Wieland and Scheuing (1920) who had finally established the nature of the reaction involved with aldehydes, and concluded that the reaction took place with double bonds in other substances besides aldehydes. It is an addition reaction of the type:



He disagrees with the Feulgen school (Feulgen, Imhäuser, and Behrens, 1929), who claimed that the reaction is a pseudo-reaction with acetone; and he states, in contradiction of Verne (1929*a*), that oleic acid does give a positive reaction, though only after 15–45 minutes. He makes two very important comments: firstly, substances which are not aldehydes and yet give a true reaction will also react with sodium bisulphite, phenylhydrazine, and other reagents supposed to identify aldehydes, and secondly, that the reaction of any such substance with one of these was no guarantee that it would react with others. Consequently, if an unidentified substance in tissues reacts both with Schiff's reagent and with phenylhydrazine, it is not characterized thereby as an aldehyde, nor does it follow that it must react with, say, semicarbazide. This paper is of the highest importance in the interpretation of results obtained with Schiff's reagent.

Lison also remarked that Schiff's reagent could be recolorized by oxidant

enzymes if oxygen were present. He was not sure of the interpretation of the colours produced, and thought that the process was probably a pseudo-reaction. Gérard (1935) studied the distribution of plasmal and of 'oxidases' (apparently peroxidases and phenolases according to Lison, 1936*b*, Chapter VI). He used dimedone (dimethyl dihydroresorcinol) to block all aldehyde radicles but found that it did not block the plasmal reaction. Further, there was a complete parallel between the plasmal reaction and the reactions for 'oxidases' except in myelin, and he found that Schiff's reagent was readily recolorized by iodates, periodates, and other oxidizers. As phenylhydrazine acetate does block plasmal, and as the Nadi reaction was positive even after treatment with cyanide (which would seem to rule out an oxidant enzyme) he inferred that there was an oxidizing agent in the lipoids; this was confirmed by the observations that oxidized oleic acid would liberate iodine from slightly acidified potassium iodide (as would sections) and would recolorize some of the reagents for 'oxidases'. Myelin explicitly excepted, his conclusion is (p. 278) that the plasmal reaction when given by inclusions shows a secondarily acquired oxidizing system.

Lison (1936*a*) also studied the Nadi reaction, and noticed that some fats give a feeble positive result with it, which he considered to be due to a direct reaction as it was not inhibited by cyanide, and was improved on boiling. He confirmed Gérard's findings, and added that the substance concerned would give a positive result with benzidine and a peroxidase, and so must be a peroxide. It is formed by atmospheric oxidation at a double bond.

Verne had shown (1937*a*) that in the kidney of the dog there were lipoid bodies that were positive with plasmal, but negative with Nadi, and that tissues which gave a coloration with Schiff's reagent when 'fresh' and after formaldehyde-fixation and treatment with mercuric chloride, did not do so if mercuric chloride were not used. Formaldehyde, in fact, would block the recoloration. He answered the objections raised by Gérard and by Lison, in two papers (1937*b*, 1940) repeating the results just mentioned, and adding that osmium tetroxide, and iridium, platinum, and gold chlorides could be used after formaldehyde-fixation as well as mercuric chloride. (Oster and Schlossman (1942) appear to have discovered the use of gold chloride independently.) His conclusion was (1937*b*, p. 276) that the Nadi reaction demonstrated the progress of autoxidation of the lipoids, whereas the plasmal reaction showed the appearance of products resulting from this autoxidation, which took place at double bonds. Consequently, the lipoids showing only the plasmal reaction ('Feulgen–Verne reaction') must be saturated, all the double bonds being destroyed. Finally he remarks that aldehydes have been detected in rancid oils.

In his second paper (1940) he showed that the plasmal reaction was inhibited by carbon monoxide and cyanide but not by ethyl-urethane. Sections of adrenal and kidney in contact with ethyl-urethane for 24 hours gave a positive reaction as if they had been in water. The reaction could be regenerated after cyanide by mercuric chloride. He thought, therefore, that the oxidation

must be mediated by a polyphenol-oxidase. As Lison had shown that the Nadi reaction was unaffected by cyanide, this strengthened Verne's views on the separate natures of the two reactions. Further, the plasmal reaction was blocked by dimedone and could not be regenerated by mercuric chloride. This result was in complete contradiction of Gérard, and Verne considered it as disposing of Gérard's objections. He also claimed that he had obtained on at least one occasion a reduction of ammoniacal silver nitrate by lipoid inclusions, which he took to demonstrate with certainty the presence of aldehyde groups.

Methods and Interpretations

The results of the histochemists are indeed far from presenting the same simplicity, clarity, and coherence as those of the biochemists; nor do they lead to the same conclusions. Also, the technique employed has been varied considerably. There appear to be several distinct reactions, which may or may not have the same basis. The reader may find it convenient at this stage to turn to the table on p. 419. The techniques have been summarized by Cain (1949).

The plasmal technique (that of Feulgen and his colleagues) appears unexceptionable, but unfortunately its users attached sections to slides with albumen by allowing them to dry, and then 'fixing' by passing through a flame. In view of Verne's conclusions on the importance of oxidation at double bonds, it might be objected that the very wide distribution of plasmal as found by the Feulgen school is due at least in part to their method of attaching their sections, provided that the oxidation, once started, is catalysed by mercuric chloride, and by acidity. Also there is the possibility of interference by oxidizers of various sorts as shown by Gérard, and by Lison (1936*a*), provided they are not destroyed by the heating.

The Feulgen–Verne technique has the great disadvantage that there is no control section. Consequently it cannot distinguish between those aldehydes or oxidizers (if any) already present in the tissues and those revealed after the action of mercuric chloride. Yet plasmal is described by its discoverer as being liberated under the influence of mercuric chloride from acetal-phosphatide. It follows that the F–V technique cannot be called a 'plasmal' technique.

The method of Gérard would appear to be the best were it not for Verne's very disturbing observation that formaldehyde can block a reaction, present in 'fresh' tissue, which can be revealed again by mercuric chloride. It appears that when discussing this (1937*a*, p. 4) he is referring to a recoloration of Schiff's reagent by fresh tissue without the intervention of mercuric chloride. The effect would be to produce a pseudo-plasmal reaction, because if the tissue were really positive when fresh and there was no intensification of the reaction after treating it with mercuric chloride, then the presence of plasmalogen in Feulgen's sense could not be demonstrated for certain; yet it would appear to be present after fixation. However, the 'plasmal reaction'

described by Liang (1947) as being produced by 'fresh' myelin occurs only after several hours' soaking in Schiff's reagent.

The interpretations placed on the different techniques vary correspondingly. The difficulty in evaluating them lies in the fact that lipoid droplets in tissues are never pure and may give rise to a variety of reactions, few of which are really connected. The best evidence is obtained when a reaction is prevented by preliminary treatment with a certain reagent, but there is no guarantee that the reagent is not reacting with other radicles or components at the same time.

Feulgen's interpretation is simple. Acetalphosphatides are decomposed, extremely rapidly with mercuric chloride, to give palmital, stearal, and other higher aldehydes, which give a positive result. Acetaldehyde, studied in urine by Stepp and Feulgen (1921, 1922), occurs in too small quantities to give positive results. It should be noted here that many authors speak of a positive result as the recolouring of Schiff's reagent. This is perfectly true in one sense—the reagent is bleached as it is prepared and now is coloured again—but very misleading in another—the colour is not necessarily or usually the same as that of the reagent recolorized on evaporation of sulphur dioxide, that is, of basic fuchsine. However, too great a reliance on the difference in shade is inadvisable. Lison (1932) showed that very various shades were given in quite genuine reactions; certain authors insist that a positive result is always much bluer than a recolouring produced merely by evaporation; but this is not always true, and in addition, the colour can vary according to the mode of viewing. A section which appears magenta when looked at under the microscope by transmitted light, may appear deep violet when lying on the bench.

Feulgen and his school were able to produce the plasmal reaction with mercuric chloride *in vitro*; and they remark frequently (e.g. Feulgen and Bersin, 1939) that the speed of reaction with mercuric chloride is very high—*fast augenblicklich*. Yet nearly always the reactions described by the histochemists require many minutes to develop, even in the presence of mercuric chloride. Feulgen and Bersin (1939) note that the speed depends to a great extent on the degree of dispersion of plasmalogen; but one would expect to find it in a state of very fine dispersion in the tissues, especially since phospholipines are extremely good dispersing agents. The comparative slowness of the reaction in tissues is very suspicious, particularly in combination with Verne's remark that it is necessary to fix for at least six hours; and the possibility of atmospheric oxidation increases with the time spent in manipulating the tissue. A reaction *fast augenblicklich* has not been described by anyone but Feulgen and his collaborators. One wonders whether a genuine and unequivocal plasmal reaction has ever been seen in fixed tissues.

Verne's great contribution has been to show that a positive reaction with Schiff can be provoked by means of oxidizing agents, and prevented by their continued action. Oleic acid, if exposed to the air, will become positive, as will triolein. And Gérard and Lison have shown that it will then

recolorize other leucobases, and that similar substances in tissues definitely appear to form peroxides. Verne maintains, however, that aldehydes are in question. All three agree that the reacting radicle—whichever it is—arises from the double bonds of unsaturated substances. One point requires explanation here. Verne, basing his statements upon the researches of Kaufmann and Lehmann (1926*a*, *b*), remarks (1937*a*, *b*) that osmium tetroxide and sudan III colour only unsaturated lipoids. This is only partly true. Osmium tetroxide will not colour directly many compounds (such as cholesterol and lecithin) which contain double bonds, although it may do so after subsequent treatment with alcohol. Sudan III and the other sudan lipoid-colorants will (as remarked above) colour all lipoids, saturated or unsaturated, provided only that they are neither combined with proteins in such a way as to mask their lipoid nature, nor are they solid—a point which is of great importance here.

It can be shown very easily that sudan black will dissolve in tristearin (for example) *if the tristearin is liquid*. But as the lipoids that are liquids or greases at room temperature, and occur at all commonly in tissues, are invariably unsaturated, Verne is right in practice although theoretically wrong. The reason why he observed lipoids becoming sudan-negative on oxidation would appear to be that the melting-point is being raised by an increase in their degree of saturation.

The various theories may be summarized as follows, the positively reacting substance being italicized:

A. *Feulgen*

Acetalphosphatide —(mercuric chloride)→ glycerophosphoric esters +*aldehydes*

B. *Verne*

(i) With mercuric chloride fixation

Unsaturated linkages —(oxidizing enzyme)→ *aldehydes*

(ii) With formaldehyde fixation

(*a*) (Unsaturated linkages —(oxidizing enzyme)→ aldehydes)

(*b*) Aldehydes + formaldehyde —(mercuric chloride)→ Labile complex

(*c*) Labile complex → *aldehydes* (+*formaldehyde* presumably)

C. *Gérard* (myelin excepted)

Unsaturated linkages + atmospheric O_2 → *oxidizer* (a peroxide according to Lison).

Feulgen's theory has the support of his very impressive biochemical researches on plasmal and plasmalogen, culminating in their preparation in the pure state, and synthesis. From the emphasis he places on the necessity for a control section it would appear that he obtained positive results with fresh tissues, but did not regard them as necessarily showing plasmal; in this he was right, because what is specific in his technique is the *rapid appearance* of positive substances after the action of mercuric chloride (or of acid solutions for a much longer period). Since a mere recoloration of Schiff's reagent is not specific for aldehydes, nothing can be said about a positive result in fresh, untreated tissue.

Verne suggested (1937*b*, p. 275) that in some cases in which the reaction is negative with fresh tissues the fixative might break down a lipoprotein complex. Where the fresh tissue is positive, he considered that aldehydes are concerned, and that these had been formed already in the tissues from unsaturated linkages, by the same method as he had used to produce a positive result after fixation, that is, by oxidation. The production of positive results with the Nadi reagent and leucobases he interpreted at first (1937*b*) as merely showing that auto-oxidation was proceeding, but later (1940) as showing the presence of an enzyme (polyphenoloxidase) catalysing the reaction.

According to Lison (1936*b*, Chapter XVI) the subject of enzymes is one of the most confused in histochemistry. What is certain is that those enzymes that can be demonstrated cytologically catalyse the oxidation of polyphenols, polyamines, and other substances that are not themselves found in tissues; and there is no evidence whatever that such enzymes will catalyse the oxidation of other substrates. They cannot catalyse normal physiological oxidations. He adds that on the other hand one cannot say that these enzymes have nothing whatever to do with cellular respiration, because 'dans ce domaine, l'obscurité est encore totale'. Verne's evidence for the action of an enzyme (namely that the plasmal reaction is blocked by carbon monoxide and by cyanide but not by ethyl-urethane) is insufficient. It is necessary to show that these substances do not by themselves prevent the oxidation of double bonds. It is at least likely that, being themselves unsaturated, they can form addition compounds at these linkages, so protecting them from atmospheric oxygen, *in vitro*.

It seems a little unjustifiable, also, to assume that a positive result in fresh tissues is necessarily due to the formation of aldehydes by the oxidation of double bonds. So very little is known of the intermediate metabolism of fatty radicles; what is known chiefly concerns the β- or ω-oxidation of saturated acids. Smedley-MacLean (1943) considered that there is no evidence that the unsaturated acids represent stages in oxidation of fatty acids, although her view is contested by some (see the review in Hilditch, 1947). It is never possible for the histochemist to anticipate the biochemist in identifying unknown radicles in tissues unless he can prove his reagents to be specific.

The specificity of the plasmal reaction depends on the catalytic action of mercuric chloride, and the specificity of the reagents used to produce or block

it, namely, phenylhydrazine and derivatives, semicarbazide and derivatives, sodium bisulphite, carbon monoxide, cyanide, dimedone, ammoniacal silver nitrate, and of course Schiff's reagent. The reaction of phenylhydrazine, semicarbazide, and their derivatives is a condensation, an intermediate addition product which is unstable being usually postulated,

$$R{-}CHO + \underset{\text{phenylhydrazine}}{H_2NNHC_6H_5} \longrightarrow RCH{=}NNHC_6H_5 + H_2O$$

$$R{-}CHO + \underset{\text{semicarbazide}}{H_2NNHCONH_2} \longrightarrow RCH{=}NNHCONH_2 + H_2O,$$

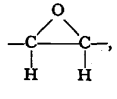
whereas the reaction with sodium bisulphite is a simple addition,

$$R{-}CHO + NaHSO_3 \longrightarrow R{-}CH(OH)SO_3Na.$$

Hydrogen cyanide, always present in solutions of its salts, will react similarly,

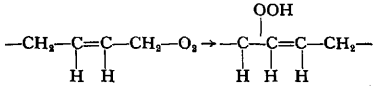
$$R{-}CHO + HCN \longrightarrow R{-}CH(OH)CN,$$

so that the blocking of the plasmal reaction by cyanide need not require the inactivation of an enzyme. These additions are reminiscent of the additions of Schiff's reagent to double bonds, and in fact sodium bisulphite will react with some >C=C< linkages (see e.g. Hickinbottom, 1948). It might be thought that the phenylhydrazine or semicarbazide condensations, which apparently require a C=O linkage might be specific. Unfortunately, these compounds are also strong reducing agents and soluble to some extent in lipoids. Their presence might well prevent the continuation of oxidations in the same way as do carotenoids. The ammoniacal silver nitrate reagents react, of course, with many reducing agents, of which aldehydes are by far the most likely to occur in lipoid inclusions. Unfortunately ethylene oxide groups (Markley, 1947, p. 461) will also reduce them, and the occurrence of such groups has been claimed (e.g. by Szent-Györgyi, 1924) in the oxidation of certain acids, particularly under the influence of —SH groups, which are the active groups in glutathione. It is not claimed that ethylene oxide groups, which have the constitution:



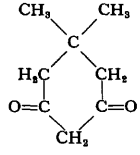
are commonly formed, but their presence must be taken into account. Research on the modes of atmospheric oxidation of unsaturated fatty acids has been carried out principally on oleic, linoleic, and linolenic acids. The very extensive and controversial literature is summarized and discussed by Hilditch (1947) and Markley (1947). Arachidonic acid has been found to occur widely in small quantities, particularly as one of the fatty acid radicles in phospholipines. Ault and Brown (1934) found that it formed over 20 per cent. of the acids in phospholipines from ox adrenal. Unfortunately no studies of its autoxidation appear to be available; as the most highly unsaturated acid present in many animal tissues, it should be of considerable importance in the plasmal reaction if Verne's theories are correct. In general, autoxidation

of oleic and linoleic acids at room temperature appears to begin by the formation of a hydroperoxide group on a carbon atom next to the double link.



This is itself an oxidizer. Further stages involve the opening of the double bond to give keto and hydroxy groups, and finally the chain breaks, giving aldehydes and diacids, the aldehydes then oxidizing to acids as well. Many of the intermediate compounds are unstable and difficult to isolate, and their properties are but little known. The use of heavy metals and their salts in hastening oxidation, probably at first by destroying anti-oxidants (most likely polyphenols), is well known in industry.

If then the unsaturated acids in tissues oxidize readily, it is likely that the hydroperoxide, keto- and hydroxy-radicles, and possibly ethylene oxide groups may all coexist with double bonds. The hydroperoxides could be expected to recolour Schiff's reagent by oxidation and reduce ammoniacal silver nitrate, and to be destroyed by the reducers phenylhydrazine and semicarbazide, which could also prevent further oxidation. It is quite possible that sodium bisulphite might act in the same way, and perhaps add on to double linkages in highly unsaturated chains, as might carbon monoxide and cyanide. Dimedone, which has been used with contradictory results by Gérard and by Verne (1940) is 5 : 5 dimethyldihydroresorcinol (1 : 1 dimethylcyclohexandione 3 : 5) and has the constitution:



According to Heilbron and Bunbury (1934) it is used for characterizing carbonyl compounds, in which case it should react with aldehydes and ketones, the latter being present in partly oxidized unsaturated acids. It also appears to be a reducing agent.

Recently proposed Techniques

If the oxidation could be catalysed by mercuric chloride, then all the reactions described by cytologists could be accounted for without the necessity for postulating enzymes, or the formation of aldehydes, or the presence of acetal linkages, unless the speed of the plasmal reaction could be shown to be too great to be explained by oxidation. In that case we have two possibilities: either there is a catalysing enzyme also present in tissues, or else the reaction is a true plasmal reaction in Feulgen's original sense.

This question has been investigated recently (Cain, 1949), with the result that a sharp distinction has been drawn between the true Feulgen plasmal reaction and the Feulgen–Verne reaction. The former is due, as Feulgen showed, to the release of higher aliphatic aldehydes (plasmal), presumably from acetalphosphatides, under the catalytic influence of mercuric chloride. Lengthy fixation *reduces* the reaction progressively, and finally abolishes it entirely. A control, uninfluenced by mercuric chloride, is essential. The Feulgen–Verne reaction *increases* in intensity with the length of time in fixative (or other fluids) provided that air has free access; it is not appreciably catalysed, or only slightly, with mercuric chloride; and it appears to be due to oxidation products, perhaps hydroperoxides, formed at points of unsaturation in fatty acid radicles by the action of atmospheric oxygen (or in sterols according to Shear and Kramer, 1926). The evidence brought forward by Verne that aldehydes are responsible rests on the assumptions that all the positive reactions used to prove this point are caused by a single substance in the tissues, and that the reactions used are specific for aldehydes, both of which assumptions are unjustified.

The investigations of Hayes (1947, 1949) support the conclusions reached by Cain, and this author especially emphasizes the necessity for a control section and for rapid handling of the tissues. He finds that formaldehyde-fixation 'rapidly dimishes and finally destroys' the true plasmal reaction and progressively develops a secondary reaction with Schiff's reagent that is not affected by mercuric chloride, and shows a quite different distribution from the true plasmal reaction in some tissues. He concludes that it 'probably demonstrates carbonyl-lipids other than acetals'. This is not quite in agreement with Cain's results, which suggest that other groups, equally produced by autoxidation, may be concerned, and that there is no direct evidence at all that carbonyl groups are the main cause, but the disagreement is slight. The results of these two workers, obtained completely independently, are in substantial agreement.

Hayes prescribes a technique involving the use of frozen sections, which are very inconvenient with some tissues. In Cain's technique (1949, p. 79) this difficulty is overcome by the use of very small pieces of tissue which are exposed while fresh to the action of Schiff's reagent, both with and without mercuric chloride, and then plunged into formaldehyde. The resulting formaldehyde-Schiff compound can be washed out, leaving the plasmal-Schiff compounds in place. Fixation by this method is not good, but it does allow of precise localization of the plasmal reaction within tissues. Penetration by Schiff's reagent is poor, and only very small pieces of tissue can be used.

The investigations of Danielli (1949) do not show results in accordance with those just described. The technique proposed by him (p. 68) is open to serious objection in that the fixative used (and recommended, p. 70) contained acetic acid, and that the time of fixation is given as not less than 2 hours and not more than 5 days. The use of acidic fixing fluids must be avoided in view of Feulgen and Bersin's remark (p. 413 above) that acetal linkages are destroyed

in acid media. The effects of long fixation are invariably bad (Hayes, 1947, 1949; Cain, 1949). Further, it does not appear that control sections were used.

Danielli states (p. 70) that the intensity of the aldehyde reaction and the nature of its distribution in liver sections appeared to be independent of the 'physical nature of the fixative', after a considerable variety of fixatives had been tried, and that variation in the times prescribed for the stages of his technique had little effect (p. 72). This suggests, in view of the criticism just made, that it was the pseudoplasmal reaction that was observed, not the true plasmal reaction.

The supplementary tests used by Danielli to support the results of his technique are:

(i) extraction of sections with fat-solvents (to demonstrate the lipoid nature of the aldehyde);

(ii) use of azobenzene phenylhydrazine sulphonic acid, which is stated to develop a purple colour with aldehydes;

(iii) use of ammoniacal silver nitrate solution, which is reduced by aldehydes;

(iv) use of 2 : 4 dinitrophenylhydrazine, which forms a yellow hydrazone with aldehydes;

(v) treatment with hydroxylamine, before carrying out the Danielli technique for aldehydes. This, by forming an oxime with the aldehydes, prevents the formation of the aldehyde-Schiff compound.

The results of these tests are considered to prove conclusively that the colour obtained with reduced fuchsin in liver sections is due to the presence in the sections of lipoidal aldehydes. But as it has been shown (Cain, 1949) that the pseudoplasmal reaction is easily prevented by the use of reducing agents which protect unsaturated fats from atmospheric oxidation, and the reagents used in tests (ii), (iv), and (v) are reducing agents, these tests cannot be used to discriminate between a plasmal and a pseudoplasmal reaction. A positive result with test (iii) might possibly be due to the presence of other reducing agents in the tissues, and is given by ethylene oxides (Markley, 1947) which may appear in the pseudoplasmal reaction. Test (i) will of course prevent the pseudoplasmal reaction as well as the plasmal. One cannot regard this group of tests as providing support for Danielli's technique.

Summary

1. The acetalphosphatides which are the precursors of the aldehydes ('plasmal') responsible for Feulgen's plasmal reaction are extremely labile compounds which hydrolyse very rapidly in acid media, and are destroyed more or less rapidly during fixation. The liberation of aldehydes from them is catalysed by mercuric chloride.

2. Ordinary unsaturated lipoids, if exposed to air, become capable of producing a colour with Schiff's reagent, which, unless a control section is

used, can be misinterpreted as a positive result for Feulgen's plasmal reaction. Mercuric chloride has little or no influence over the oxidative rancidity of unsaturated lipoids.

3. Any technique prescribed for showing the plasmal reaction must avoid acid media and prolonged fixation and handling of the tissues. A control section is a necessity.

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