The Nature of a Chromosomal Phospholipid

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SUMMARY

It has been found that the heterochromatic region of interphase nuclei of plant roots and calf thymus, fixed in formaldehyde solution, do not stain for phospholipid as they do after Lewitsky's fixative. It has been shown that this is probably due to the extraction of a protein-phospholipid complex. Some lipid-like material has been purified from the used formalin and shown to contain glycerophosphate. The nature of the association between the lipid and protein is discussed.

INTRODUCTION

WHEN plant roots were fixed in Lewitsky's (1931) fluid and embedded by the normal procedures, the mitotic chromosomes and the heterochromatic regions of the interphase nuclei gave apparently positive reactions for phospholipids by the acid haematein test and stained yellow in a mixture of orange G and aniline blue (La Cour, Chayen, and Gahan, 1958; compare Baker, 1946). If, however, the tissues had been fixed first in formaldehyde-calcium (Baker, 1946) and then in Lewitsky's solution, neither the mitotic chromosomes nor the interphase heterochromatic regions reacted with the acid haematein and they stained blue in the double stain. On the other hand, when such tissues were treated with hot pyridine, hot ethanol-ether, or with 5% trichloroacetic acid at 90° C for 15 min, the whole of the interphase and mitotic chromosomes gave an intense reaction with the acid haematein method and stained yellow in the dye mixture. It might have been thought that the difference in staining produced by prefixation in formaldehyde-calcium indicated that this solution had removed a chromosomal phospholipid. The objection to this conclusion was that, after such fixation, methods which were believed to remove lipids produced enhanced, not reduced, staining, so casting doubt on the validity of the histochemical reactions for phospholipids. The problem of the enhanced staining formed the basis of a separate investigation (see Chayen, Gahan, and La Cour, 1959). The question whether the difference in staining behaviour after the two fixations could have been due to some solvent action of the formaldehyde-calcium forms the subject of the present study.

We have included some results obtained with calf thymus. In this tissue the nuclei, when fixed in Lewitsky's fluid, stain uniformly yellow in the double-stain and react positively to acid haematein. As in roots, these reactions are inhibited by formaldehyde-calcium fixation.

**MATERIAL AND METHODS**

**Material.** The terminal 5–10 mm of roots of *Trillium grandiflorum* grown in soil in pots and of roots of *Vicia faba* grown in damp filter paper were used in this investigation. Calf thymus was also studied: the gland was removed from the animal immediately after slaughter and packed in dry ice for transportation to the laboratory.

**Methods.** The technique for purifying and hydrolysing phospholipids and for their chromatographic analysis are described fully in another communication (Chayen, Gahan, and La Cour, 1959). The staining methods were the modified acid haematein and the orange G aniline blue procedures used by La Cour and others (1958), as well as the usual alcoholic Sudan black method. The fast green technique of Alfer and Geschwind (1953) was also studied; deamination was performed by leaving sections at room temperature for 10 min in a solution composed of equal volumes of 0.1 N hydrochloric acid and 0.1 N sodium nitrite, which were mixed immediately before use.

**RESULTS**

The materials to be studied, namely 200 root tips of *T. grandiflorum*, 1500 of *V. faba*, and small pieces of calf thymus, were fixed for 6 h in formaldehyde-calcium and were then transferred to fresh fixative for a further 24–48 h. Some roots were studied cytologically to see whether the mitotic and interphase chromosomes gave any reaction for phospholipids. The used fixatives were centrifuged to remove solid matter and, if not completely clear, they were filtered under reduced pressure through a Seitz filter. When completely clarified, their ultraviolet absorption was examined in a Beckman spectrophotometer and they were then shaken with equal volumes of chloroform. A heavy milky precipitate formed which, on standing, came to rest at the interface between the chloroform and the fixative. This precipitate was removed together with the chloroform and the fixative was again shaken with fresh chloroform, this procedure being repeated until almost all the precipitable material had been removed. The ultraviolet absorption of the residual formaldehyde-calcium was tested. Since the behaviour of the matter extracted by the long fixation was the same as that by the short treatment, the two precipitates usually were combined.

It was then necessary to dissolve in methanol-chloroform (1:2, v/v) any phospholipid present in the precipitate. In the large-scale experiments with calf thymus most of the chloroform was drained off first; with the plant material, which was obtained in small quantities only, the chloroform was retained and a suitable volume of methanol was added to it. The precipitate was shaken gently with a large volume of methanol-chloroform and was allowed to stand at room temperature for about 1 week. During this time, much of it dissolved, leaving a white flaky or fluffy residue which had to be separated from the clear solution. The absorption characteristics of the clear solution were examined. The fluffy residue obtained from calf thymus was hydrolysed with 6 N hydrochloric acid for 5 h and on chromatographic analysis was found
to contain a large number of amino-acids. These were difficult to resolve by the methods used, but spots having the $R_F$ value of arginine, serine, and possibly ethanolamine were seen. Free phosphate, and some material which reacted like choline but which did not run free were also detected. These results would indicate that this residue which was insoluble in methanol-chloroform might consist of a protein which was associated with a lipid-like substance, possibly of a type resembling sphingomyelin.

The solution in methanol-chloroform was subjected to the procedure of Folch (see Chayen, Gahan, and La Cour, 1959). As the methanol escaped into the water the solution containing the suspected lipid became milky and finally, when all the methanol had diffused into the water, a white milky precipitate formed at the interface between the chloroform and water. The chloroform and the precipitate were redissolved in methanol-chloroform and the procedure was repeated with the same results.

A suitable volume of methanol was again added to the chloroform to dissolve the precipitate and the absorption spectrum of the solution was examined. The solvent was evaporated off; some of the residue was examined microscopically, while the rest was hydrolysed in alcoholic potash and investigated by partition chromatography. No choline, serine, ethanolamine, or inositol were found, although slight traces of other amino-acids were seen occasionally. Glycerophosphate was identified repeatedly. In other tests (by the method of McKibbin and Taylor, 1949) no sphingosine could be detected.

Histochemical tests were performed on the precipitates. The matter which was precipitated out of the fixative by shaking it with chloroform gave strong reactions for arginine by the method of McLeish and others (1957); both this material and the purified lipid-like matter yielded positive reactions with the acid haematein and the orange G aniline blue methods. When the lipid-like material was dried from solution in methanol-chloroform it formed an almost crystalline pattern which altered drastically on wetting.

When root tips, or calf thymus, were fixed in Lewitsky’s fluid and the used fixative shaken with chloroform, no precipitate was formed. Similarly, no precipitate was obtained from the used fixative when a methanolic solution of ammonium reineckate was added to it; this reagent produced a heavy precipitate when added to formaldehyde-calcium which had been used to fix plant roots.

Since the material extracted by formaldehyde-calcium yielded a strong reaction for arginine, it was advisable to study the effect of this fixative histochemically. Sections of roots of *T. grandiflorum* and of *V. faba* which had been fixed in either formaldehyde-calcium or in Lewitsky’s fluid were tested with the modified arginine reaction (McLeish and others, 1957). The nuclei of those fixed in Lewitsky’s solution stained more intensely, especially in the heterochromatic regions, than did those treated with formaldehyde-calcium.

An attempt was made, therefore, to use the fast green method, which is said to be specific for basic proteins of the histone type (Alfert and Geschwind, 1953). The dye was dissolved at about pH 8, the pH being adjusted...
either by the addition of alkali or by adding the dye to a citrate-phosphate buffer solution at this pH. The nuclei in sections of roots which had been fixed in the chromic fixative stained very much more intensely than did those which had been treated with formaldehyde-calcium. Staining after either fixation was stronger with the buffered than the unbuffered solution, and was largely removed if the tissue was first deaminated.

**DISCUSSION**

The material removed by formaldehyde-calcium. It is clear from the absorption curves in Chayen, Gahan, and La Cour (1959) that formaldehyde-calcium removes from calf thymus and from *Trillium* roots some material which resembles nucleic acid or nucleotides (peak about 260 m\(\mu\)) and protein (peak about 280 m\(\mu\)). After it has been shaken with chloroform, almost all the nucleotide-like matter is left in the fixative but much of the substance absorbing maximally at about 280 m\(\mu\) has been precipitated out of solution. This precipitate stained with the arginine reaction; similarly after this fixation, the interphase nuclei stained less strongly for basic proteins and for arginine than they did after fixation in a fluid from which no such precipitate was obtained.

Histochemical data indicated that formaldehyde-calcium, but not Lewitsky's fluid, removed matter particularly from the heterochromatic regions of interphase nuclei and from mitotic chromosomes. The results with the arginine and the fast green methods suggested that formaldehyde-calcium removed a protein. The loss of staining with the acid haematein and the orange G aniline blue procedures after this fixative were less certain indications of the simultaneous removal of a phospholipid from the same sites owing to doubts concerning the validity of the techniques. It has now been shown that from the used formaldehyde-calcium, but not from used Lewitsky's solution, matter can be precipitated which may be resolved into two components, depending on their solubility in methanol-chloroform. The insoluble material, on hydrolysis, yielded chromatograms which confirmed the view that formaldehyde-calcium removed protein; there was some indication that it was closely associated with some lipid-like substance resembling sphingomyelin. The soluble matter seemed to contain traces of substances resembling nucleotides (curve (iii), fig. 1, B, in Chayen, Gahan, and La Cour, 1959). These must have been intimately associated with lipid in that they were soluble in methanol-chloroform but they were largely removed by the prolonged Folch procedure. The material that remained confirmed the histochemical results for phospholipid in that it yielded glycerophosphate after hydrolysis, but it was exceptional in that no choline, serine, ethanolamine, or reducing sugars were detected. It is unlikely to be free glycerophosphate owing to the nature of its isolation. Hence, despite the lack of proof for the presence of fatty acids, it must be considered tentatively to be either a phosphatidic acid or a phospholipid which resembles lecithin in containing glycerophosphate but in which the choline is replaced by an unknown nitrogen base. Determination of the
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ratio of phosphate to nitrogen did not clarify the problem since varying amounts of protein or peptide compounds were left after the Folch procedure, as is observed frequently when true lipo-proteins are subjected to this treatment.

The nature of the lipid and protein. It seems possible, therefore, that as was suggested by the histochemical data, formaldehyde-calcium removes from calf thymus and from roots of *T. grandiflorum* and *V. faba* a phospholipid and a protein which may have a high proportion of basic amino-acids. The evidence indicates that they are removed as a complex which is split by the later treatment. It should be emphasized that, in agreement with Baker (1946), no other phospholipids were extracted by this fixative as far as could be detected. The problem, therefore, is why this phospholipid should be the exception and why formaldehyde has extracted a protein.

It may not be unreasonable to suggest that this problem could be resolved if the phospholipid and protein were associated in such a way that the lipid protected the free amino-groups of the protein against fixation by formalin while the protein rendered the complex soluble in an aqueous medium. This might be achieved, for example, if a phosphatidic acid were esterified with such groups as the guanido-amino of arginine. It is noteworthy that Barnard and Danielli (1956) found that histidine which was present in nuclei, and predominantly in the heterochromatic regions (Barnard, personal communication), was protected against benzoylation by combination with an unknown substance which was thought to be deoxyribonucleic acid. It seems possible that their results might have been caused by the combination of the amino-acid with the lipid-like substance reported in the present communication.

The evidence that the complex is present on chromosomes. When plant roots or mouse liver are fixed in Lewitsky's fluid, the heterochromatic regions of interphase nuclei and the mitotic chromosomes stain for phospholipid but they fail to do so when fixed in formaldehyde-calcium (see La Cour, Chayen, and Gahan, 1958; Chayen, Gahan, and Harvey, in preparation). A lipid-protein complex has been isolated from the latter, but not from the former fixative after use, so confirming the gross histochemical findings. Two questions remain to be answered; the first concerns the reliability of the staining procedures, and the second the possibility of a diffusion artifact in the histochemical localization of the lipid.

(i) The validity of the staining methods has been examined and discussed elsewhere (Chayen, Gahan, and La Cour, 1959; La Cour and others (1958). Moreover the same distribution of lipids has been observed when benzpyrene, a fluorochrome, has been used after these fixatives (Chayen and others, 1957). While it might be argued that the acid haematein method is staining some interfering substance, there can be no doubt that benzpyrene concentrates only in lipid material (Berg, 1951).

(ii) When fixation was effected by formaldehyde-calcium containing ammonium reineckate, a precipitant of this lipid-protein complex, the distribution of phospholipid was identical with that found after Lewitsky's fixation.
On the other hand, obvious diffusion and precipitation artifacts were observed when this salt was added to methanol that was used for fixation (La Cour and others, 1958). In addition, benzpyrene has been localized in heterochromatic regions of interphase nuclei of living macrophages from mouse lung grown in tissue culture, which suggests that these regions contain lipid in life (Chayen, Gahan, and Harvey, in preparation).

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REFERENCES