1-FLUORO-2:4-DINITROBENZENE AS A CYTOCHEMICAL REAGENT

By A. H. MADDY

Zoology Department, University of Edinburgh

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>259</td>
</tr>
<tr>
<td>II. Theory of Method</td>
<td>261</td>
</tr>
<tr>
<td>A. The Reactivity of FDNB and Benzoyl Chloride</td>
<td>261</td>
</tr>
<tr>
<td>B. The Effect of Fixation</td>
<td>267</td>
</tr>
<tr>
<td>C. Detection of the Nitro Groups in Tissue Sections</td>
<td>269</td>
</tr>
<tr>
<td>D. The Water Abolition Effect</td>
<td>269</td>
</tr>
<tr>
<td>E. The Relationship between the Reaction of FDNB and that of tetrazotized dianisidine</td>
<td>271</td>
</tr>
<tr>
<td>III. Histological Procedure</td>
<td>273</td>
</tr>
<tr>
<td>A. Fixation and Preparation of Tissues</td>
<td>273</td>
</tr>
<tr>
<td>B. The Cytochemical Schedules</td>
<td>273</td>
</tr>
<tr>
<td>IV. Analytical Investigation of the Reactive Components in Nuclei</td>
<td>275</td>
</tr>
<tr>
<td>A. Preparation of Nuclei</td>
<td>275</td>
</tr>
<tr>
<td>B. Benzoylation and Dinitrophenylation of the Isolated Nuclei</td>
<td>276</td>
</tr>
<tr>
<td>C. The Fractionation of the Reacted Nuclei into their Constituent Chemical Components</td>
<td>276</td>
</tr>
<tr>
<td>D. The Identification of the Nuclear Components which React with FDNB</td>
<td>277</td>
</tr>
<tr>
<td>E. The Quantitative Analysis of the Reactive Nuclear Groups</td>
<td>278</td>
</tr>
<tr>
<td>V. Critique of the Method</td>
<td>280</td>
</tr>
<tr>
<td>VI. Results to Date</td>
<td>280</td>
</tr>
<tr>
<td>References</td>
<td>284</td>
</tr>
</tbody>
</table>

I. INTRODUCTION

The chemical complexity of protein molecules and their interactions have proved a major obstacle to their cytochemical study. Most of the methods that are available for the location of proteins in cells depend on either the determination of the distribution of biologically active protein centres, e.g. enzymic studies, and techniques using fluorescent antibodies, or the distribution of particular prosthetic groups and amino acid side chains. A third approach is based on the interaction of proteins with ionic dyes. All methods depend on the presence at the sites of protein activity of suitably absorbing chromophores. In a few cases, the proteins themselves have suitable intrinsic spectra, e.g. the ultraviolet absorption...
of the aromatic side chains of the constituent amino acids utilized by Caspersson (1947), and the absorption in the visible spectrum of the prosthetic groups of haem proteins used by Thorell (1947), in a distributional study of these conjugated proteins. The scope of this type of approach can be greatly widened by the introduction of suitable absorbing groups into the protein. Danielli (1947, 1950, 1953) has suggested that such groups may be introduced by the use of well-known protein reagents, which combine with certain of the protein side chains to form stable covalent derivatives, which are either themselves coloured, or are easily converted into coloured derivatives.

One of the reagents mentioned by Danielli was 1-fluoro-2:4-dinitrobenzene (FDNB). A consideration of the properties of this compound indicates that it is well suited for cytochemical application. The reagent has a very pale yellow colour and will not itself stain the section; excess can easily be removed from the section by organic solvents. FDNB will react with the cell components under mild conditions to form stable, covalent, indiffusable products. A cytochemical reagent should have a known specificity, but as the properties of an amino acid can be significantly altered by incorporation into a protein, a knowledge of the reactivity of an amino acid in the free state cannot be used to predict unequivocally its behaviour towards a cytochemical reagent when it is incorporated into a protein. Although the specificity of a reagent cannot be predicted with certainty, if the derivatives it forms with the cell constituents are stable, its specificity may be determined experimentally by the isolation and identification of the derivatives. The stability of known dinitrophenyl (DNP) derivatives allows the specificity of FDNB to be investigated in this way.

When a tissue section is treated with FDNB, all parts of the section react and the result is an intense overall stain. This extensive staining would be expected from the rather low specificity of the reagent, and therefore the application of FDNB after treatment of the section with a blocking agent was investigated. The blocking agent chosen was benzoyl chloride, which, as it can react with all the groups which react with FDNB, would be expected to completely block the reaction of the latter. It was actually found that certain groups in a wide variety of cell nuclei, and in a few characteristic cytoplasmic sites (Table I) were resistant to benzoylation and could react subsequently with FDNB (Maddy, 1961a). The immediate problems in the cytochemical application of FDNB were therefore:

(i) The identification of the groups which reacted with FDNB in previously untreated sections.
(ii) The identification of the residues which reacted after benzoylation.

(iii) The quantitative determination of all these groups.

(iv) The elucidation of the mechanism of the protection from benzoylation, and the investigation of the metabolic role of the protected groups.

The reactive groups in chicken erythrocyte nuclei have been identified, and the quantities present in a bulk preparation of these nuclei assessed. The mechanism of the protection from benzoylation and the significance of the protected groups remains obscure.

II. THEORY OF METHOD

A. THE REACTIVITY OF FDNB AND BENZOYL CHLORIDE

A consideration of the reactivity of a chromogenic agent with compounds present in the living cell is necessary before any investigation of its properties as a cytochemical reagent is carried out. As accurate cytochemistry is at present mostly concerned with compounds of low solubility, usually macromolecules that are not leached out of the cell during the cytochemical procedure, the cytochemist is primarily concerned with the reactivity of the chromogen with these macromolecules. However, it must always be remembered that the chromogenic reagent may react with some small, soluble molecule which has been absorbed onto the macromolecules. The relevant information on the reaction of FDNB with macromolecules present in the cell has therefore been gathered together, both from published reports and from personal observations, and is presented in this section.

1. Reaction with Proteins

FDNB was introduced as a reagent for the determination of the N-terminal amino acids of proteins by Sanger (Sanger, 1945, 1955; Porter, 1950b; Fraenkel-Conrat et al., 1955), but this much-publicized reaction must not be allowed to obscure the wide range of the reagent's activity. In addition to its reaction with α-amino groups of amino acids, it reacts with the side chains of a number of bifunctional amino acids, both when the acids are in the free state and when combined in a polypeptide chain.

a. N-terminal Amino Groups

Sanger (1945) has shown that α-amino groups react quantitatively with FDNB when the amino acids are shaken with the reagent at room temperature for 2 hr in an ethanolic bicarbonate solution. The compounds are bright yellow in colour and are easily crystallized.
b. \textit{\v{e}-Amino Group of Lysine}

As would be expected from the behaviour of \(\alpha\)-amino groups, the \(\v{e}\)-amino group of lysine reacts under similar conditions to form a bright yellow crystalline compound. Sanger (1945) has described the synthesis of the \(\text{bis}\)-dinitrophenyl derivative of free lysine, the \(\alpha\)-\(N\)-dinitrophenyl derivative from \(\v{e}\)-\(N\)-benzoyl lysine, and the \(\v{e}\)-\(N\)-dinitrophenyl derivative from \(\alpha\)-\(N\)-acetyl lysine. The \(\v{e}\)-\(N\)-derivative can be prepared by a more direct route using the copper chelate of free lysine (Porter and Sanger, 1948). It is the \(\v{e}\)-\(N\)-dinitrophenyl derivative which is obtained by the reaction of FDNB with non-\(N\)-terminal lysine. The three lysine derivatives are bright yellow.

c. \textit{The Phenolic Hydroxyl Group of Tyrosine}

Sanger (1945) found that 1-chloro-2:4-dinitrobenzene reacted with free tyrosine to form two yellow compounds—\(O\):\(N\)-\(bis\)-dinitrophenyltyrosine and \(N\)-dinitrophenyl tyrosine. \(N\)-acetyltyrosine gave a colourless derivative with FDNB, which, by the removal of the acetyl residue, was converted to the colourless \(O\)-dinitrophenyltyrosine. This latter compound can also be prepared from the copper chelate of tyrosine (Zahn and Würz, 1952).

d. \textit{Aliphatic Hydroxyl Groups, e.g. Serine}

The reaction of aliphatic hydroxyl groups with FDNB is not clearly understood: they undoubtedly react in the presence of triethylamine, but appear to be inactive when sodium bicarbonate is used as a base. Thus Whalley (1950), using triethylamine as a catalyst, has prepared the dinitrophenyl derivatives of a wide range of aliphatic alcohols. Zahn and Gerstner (1955) have synthesized \(O\)-dinitrophenylserine under similar conditions, and Zahn and Zürn (1958) have prepared the \textit{tris}-dinitrophenyl derivative of hydroxylysine. These reactions are not possible in the absence of triethylamine, and in bicarbonate no reaction between the hydroxyl groups of cotton and FDNB can be detected (Zahn and Gerstner, 1955).

e. \textit{The Sulphhydryl Group of Cysteine}

In 1934, Saunders prepared a dinitrophenyl derivative of cysteine. The compound gave a negative ninhydrin reaction, therefore it is unlikely that this compound was \(S\)-dinitrophenylcysteine. Sanger (1945) claimed to have prepared \(S\)-dinitrophenylcysteine as a hydrolytic product of dinitrophenyl glutathione, but the compound was not fully characterized. The problem has been clarified by Zahn and Traumann (1954) who have synthesized a pale yellow \(S\)-dinitrophenylcysteine by treating free cysteine with FDNB in a buffer at pH 5·2. Under these con-
ditions the sulphydryl group is reactive, but the amino group is protonated and therefore unreactive.

Leandri and Tudno (1955) have reported that 1-chloro-2:4-dinitrobenzene reacts with asymmetric but not symmetric disulphides; the disulphide bond is split and the chloro compound reacts preferentially with the more electronegative sulphur atom. In no known case has the formation of $S$-dinitrophenylcysteine from a protein been regarded as being brought about by this type of mechanism, but the possibility of such a reaction should not be excluded.

f. **Histidine**

The reaction between histidine and FDNB is not properly understood. Free histidine reacts readily with FDNB to form a bright yellow bis-dinitrophenylhistidine. $\alpha$-N-dinitrophenylhistidine has been prepared (Ramachandran and McConnell, 1955; Harnick and Margoliash, 1955), but an unequivocal synthesis of im-dinitrophenylhistidine has so far not been reported. Sanger (1945) attempted to synthesize this compound by reacting $\alpha$-N-acetylhistidine with FDNB; little reaction took place, and when the product, a yellow oil, was hydrolysed to remove the acetyl residue, two compounds were obtained. The first was not extracted from the acid solution by ether and was not detected in the hydrolysate of dinitrophenyl insulin. It was not identified. The second was probably 2:4-dinitroaniline, presumably formed by the decomposition of im-dinitrophenylhistidine.

The studies of Gerngross (1919) on the benzoylation of histidine, had already indicated the possible complexities of the problem. Gerngross found that further benzoylation of N-benzoylhistidine by benzoyl chloride was impossible, although a second benzoyl residue could easily be introduced into N-benzoylhistidine methyl ester. (The second residue is attached to the imidazole ring). The reaction of the free ester with benzoyl chloride resulted in the rupture of the imidazole ring and yielded I (Fig. 1) (Bamberger and Berle, 1893). This work suggested to Sanger the possibility of synthesizing im-dinitrophenylhistidine by reacting FDNB with $\alpha$-N-benzoylhistidine methyl ester, but he found that the reaction proceeded very slowly and the products were the same as those derived from $\alpha$-N-acetylhistidine. In contrast to the reaction of benzoyl chloride, histidine methyl ester reacted readily to form bis-dinitrophenylhistidine. In a later paper Porter (1950a) reported that Sanger had

---

1 The Cyclo Chemical Corporation (Los Angeles) has recently been able to synthesize im-N-(2:4-dinitrophenyl)-histidine by the reaction of FDNB with carbobenzoxyhistidine methyl ester and subsequent removal of the protecting groups (private communication). This DNP derivative and the DNP derivatives of many other amino acids are commercially available from the Corporation.
synthesized a colourless ninhydrin-positive material from the reaction of FDNB with \( \alpha-N \)-acetylhistidine and subsequent removal of the acetyl group. This, at the time, was thought to be \( im \)-dinitrophenylhistidine, but analysis indicated the contrary and the compound remained unidentified (Sanger, personal communication).

Zahn and Pfannmüller (1958) have re-investigated the reaction between FDNB and imidazole and its derivatives. Imidazole itself formed a pale yellow compound with FDNB, 1-dinitrophenylimidazole (II), but the product of the reaction with 4(5)-methylimidazole depends on the molar concentrations of the reactants. With a slight excess of imidazole, 1-dinitrophenyl-4-methylimidazole was formed (III), but an excess of FDNB resulted in bright red crystalline product with an elementary analysis corresponding to (IV). Zahn and Pfannmüller were not able to isolate similar derivatives from dinitrophenyl peptides, or from the reaction of \( \alpha-N \)-acetylhistidine, or \( \alpha-N \)-benzoylhistidine methyl ester with FDNB. The present author has observed the formation of a bright red compound during attempts to prepare \( im \)-dinitrophenylhistidine from \( \alpha-N \)-acetylhistidine. Zahn and Pfannmüller have also attempted to prepare \( im \)-dinitrophenylhistidine by reaction at a low pH where the
α-amino groups were protonated. The reaction was studied at both pH 4.8 and 5.8, but the desired compound was not obtained. However, a compound which is probably \( im \)-dinitrophenylhistidine can be detected when FDNB is reacted with histidine at pH 7.0, but not at lower pH values (Maddy, unpublished result).

After consideration of these difficulties, it was decided to attempt the synthesis through the cyclic anhydride of histidine. On reacting this compound with FDNB, a pale yellow product was obtained which, when hydrolysed by acid, yielded a colourless nitro compound which was ninhydrin-positive and was believed to be \( im \)-dinitrophenylhistidine. The hydrolysate also contained a yellow decomposition product. The colourless compound had the same properties as a spot obtained from dinitrophenyl-protein hydrolysates. The only known amino acid in the protein which could possibly have given rise to this derivative was histidine. In the chromatographic solvent, butanol-acetic acid-water specimens of the suspected \( im \)-dinitrophenylhistidine (whether obtained from dinitrophenylproteins—including dinitrophenylinsulin—or by preparation from histidine anhydride), were partially resolved into two components which could have been the two isomers (V) and (VI), or \( im \)-dinitrophenylhistidine and an unknown decomposition product. It would appear, therefore, that the same compound is prepared by the reaction of FDNB with histidine anhydride and with protein histidine, and, although it has not been fully characterized, it is probably \( im \)-dinitrophenylhistidine or its decomposition product.

Although the side chain of a free amino acid may be reactive, its reactivity cannot be predicted when it is incorporated into a protein. This problem is one of the major difficulties of protein chemistry, and it complicates work on the reaction of FDNB with proteins, making it impossible to forecast the reactivity of protein side chains with the reagent.

The work of Porter (1950a) gives an example of amino acid side chains which are inactivated when incorporated into a protein. He found that 50% of the histidine residues of β-lactoglobulin did not react with FDNB when the protein was in the native state, but they became reactive after denaturation of the protein. Similarly (Porter, 1948), not all the lysine residues of native β-lactoglobulin can react with FDNB although they are available in the denatured protein. In contrast, all the \( \varepsilon \)-amino groups in the native protein can be acetylated with ketene, presumably because there is less steric hindrance to the approach of the ketene molecule than to the approach of the FDNB molecule. Little purpose would be served by quoting further examples, but mention should be made of the work of Zuber, Traumann and Zahn (1955) who showed that side chains could be activated by incorporation into the protein molecule. At pH
2. Reaction with Lipids

FDNB cannot be expected to react with simple triglycerides, but Collins and Wheeldon (1955, 1958) have reported a reaction with phospholipids containing ethanolamine or serine. FDNB reacts with the free amino groups of these bases. Dinitrophenyl residues have been found in the lipid fraction of chicken erythrocyte nuclei (Maddy, 1961b).

3. Reaction with Carbohydrates

The published evidence suggests that FDNB does not react with sugar hydroxyl groups. Thus, Kent et al., (1951) state that FDNB reacts with the amino groups of amino sugars, but they do not report any reaction with the sugar hydroxyl groups. Similarly, Weygand and Lowenfeld (1950) describe the reaction of FDNB with sugar aldoximes to form unstable complexes which decompose into the next lower aldose, dinitrophenyl and hydrogen cyanide, but they do not discuss any reaction with the sugar hydroxyl groups. Zahn and Gerstner (1955) could detect no reaction between the hydroxyl groups of cotton and FDNB reacted in the presence of bicarbonate. On the other hand Pridham (personal communication) finds that on reacting glucose with FDNB in the presence of dimethylformamide and silver oxide, a colourless dinitrophenyl compound is formed. The evidence, therefore, is inconclusive, and the complexities mentioned earlier of the reaction of aliphatic hydroxyl groups with FDNB suggest the possibility of reaction under certain conditions. No evidence for the reaction of FDNB and carbohydrate was obtained from the analytical work reported in Section IV, p. 275.

4. Reaction with Nucleic Acids

No reaction of FDNB with nucleic acids has been demonstrated by previous workers, and no conclusive evidence of such a reaction has been obtained in the present investigation.

Benzoyl chloride can only function as an efficient blocking agent to FDNB if the benzoyl derivatives are stable under the conditions of
dinitrophenylation. It is known that O-benzoyl derivatives are less stable to alkaline hydrolysis than are the N-derivatives, but the inactivity of the cytoplasm of benzoylated tissues towards FDNB indicates that under the reaction conditions used, no benzoyl residue is removed to expose a site which reacts with FDNB.

The variety of groups in cells which could possibly react with FDNB, together with the extreme chemical complexity of the living cell, precludes any theoretical prediction of the actual sites that combine with FDNB. As an investigation of the identity of the reactive groups in a single tissue section is not, at present, practical by unequivocal analytical techniques, the reactivity of a reagent can only be assessed by an analysis of a bulk preparation of tissue which has been treated with that reagent by a procedure as similar as possible to the method used on the tissue sections. The reaction of FDNB with cell nuclei has been studied by reacting a bulk preparation of chicken erythrocyte nuclei with FDNB and benzoyl chloride under the conditions used during the histological investigation, fractionating the nuclei into lipid, nucleic acid, and protein components, and identifying the dinitrophenyl amino acids in the protein fraction. FDNB, when not preceded by benzoylation, reacted with the lipid fraction, the N-terminal amino acids of the protein, and the histidine, cysteine, lysine and tyrosine side chains. Benzoyl chloride blocked the N-terminal amino groups, and greatly reduced the quantities of the reactive protein side chains and lipid groups.

The bulk preparations of nuclei have also been analysed quantitatively, and the effect of benzoylation on the subsequent dinitrophenylation determined. Nuclei prepared by the standard method were benzoylated for varying periods of time, and then reacted with FDNB under standard conditions. The reacted nuclei were fractionated and the degree of dinitrophenylation of the protein estimated. As was indicated by the histological work, most of the reactive sites were found to be benzoylated after 30 min, but a significant fraction was not benzoylated, and no further benzoylation could be detected after the first 3 hr. The greater part of the nuclear reaction after benzoylation was due to tyrosine residues, amounting to about 2% of the total nuclear tyrosine available to FDNB. The lysine, cysteine and histidine side chains which reacted were present in quantities too small to be determined.

B. THE EFFECT OF FIXATION

All the tissues examined (Table I), with all the methods of fixation used, reacted with FDNB when it was applied without a blocking agent. Alcohol fixed and frozen dried sections stained intensely: the staining
### TABLE I

The Action of FDNB on Benzoylated Tissues  
(The preparations were benzoylated for 3 hr and then treated with FDNB)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Type of preparation</th>
<th>Staining of nucleus</th>
<th>Staining of cytoplasm</th>
<th>Additional features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Stomach</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Cytoplasm of submucosal band +</td>
</tr>
<tr>
<td>Rat</td>
<td>Small intestine</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Intracellular mucus +</td>
</tr>
<tr>
<td>Rat</td>
<td>Pancreas</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Goblet cells +</td>
</tr>
<tr>
<td>Rat</td>
<td>Liver</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Cytoplasm of submucosal band +</td>
</tr>
<tr>
<td>Rat</td>
<td>Kidney</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Erythrocyte nuclei +</td>
</tr>
<tr>
<td>Chick</td>
<td>Kidney</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Erythrocyte nuclei +</td>
</tr>
<tr>
<td>Chick</td>
<td>Lung</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Spleen</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Smooth muscle</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Cardiac muscle</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>FDNB is tenaciously adsorbed by the cytoplasm and it is very resistant to reduction</td>
</tr>
<tr>
<td>Rat</td>
<td>Striated muscle</td>
<td>Teased</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Ovary</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Skin (ear)</td>
<td>Frozen section</td>
<td>+</td>
<td>-</td>
<td>Nuclei of leukocytes +</td>
</tr>
<tr>
<td>Chick</td>
<td>Blood</td>
<td>Air dried smear</td>
<td>+</td>
<td>-</td>
<td>Erythrocytes not stained</td>
</tr>
</tbody>
</table>

of tissues fixed in fixatives containing formalin and heavy metals (e.g., chromic acid) was slightly less intense. These latter fixatives should be avoided in cytochemical work, as the fixative itself reacts with the protein and prevents the subsequent reaction of the chromogenic reagent.

The effect of benzoylation was markedly influenced by the method of fixation and preparation of the section. Alcohol fixed and freeze-dried, wax-embedded sections, reacted in an irreproducible manner towards FDNB after benzoylation. Sometimes a section reacted uniformly, the nuclei were all reactive and the cytoplasm was not, but usually the result was variable, with regions of complete negative staining, and on other occasions the whole section was negative. Freeze-dried material tended to be negative more often than alcohol fixed material. In contrast, smears of chicken erythrocytes and fresh frozen sections of rat and chicken tissues always showed positive nuclei and negative cytoplasm in a completely reproducible manner.

The evidence indicates that the irreproducibility was a result of the treatment of the sections before or during fixation, and not to differences
in the subsequent treatment. In the first place, wax-embedded serial sections on the same slide sometimes reacted differently, although they had all been treated identically. Secondly, the erythrocytes examined in freeze-dried, wax-embedded sections of chicken lung and kidney were completely negative, but the nuclei of air-dried smears of chicken blood cells reacted in the same Coplin jars at the same time all stained. All attempts to eliminate this irreproducibility were unsuccessful. The reaction conditions of the dinitrophenylation were varied from anhydrous to aqueous conditions, the effect of denaturing agents (acid, alkali, urea and guanidine), oxidizing (quinone) and reducing (ascorbic acid) agents, and a metal chelating agent (ethylene-diamine-tetra-acetic acid) were all studied without success. It was noted that if a denaturing agent was applied to a section after benzoylation, a weak overall reaction to FDNB was possible. This suggested that the commonly observed release of active groups in the protein by denaturing agents had occurred. (In all these experiments control sections were examined by the tetrazonium technique (Barnard, 1961), which in all cases gave uniformly positive nuclei). It therefore appeared that wax-embedding had an irreversible effect on the tissue, and it is suggested that this may have been due to the partial removal of some lipid component from the section. If the lipid was not removed, certain groups in the nuclei which reacted with FDNB were protected from benzoylation, but removal of the lipid destroyed this protection. This explanation is not wholly satisfactory, as the treatment of blood smears with molten wax and xylene did not prevent the reaction with FDNB after benzoylation; it is possible that this anomaly is due to the fact that it is impossible to simulate exactly the conditions of wax-embedding. It is interesting to note that Chayen and his co-workers (Chayen et al., 1959) have produced independent evidence for the binding of a chromosomal protein fraction to a lipid, and have already suggested that this bonding might protect the protein from benzoylation.

C. DETECTION OF THE NITRO GROUPS IN TISSUE SECTIONS

After treatment with FDNB, a section has a bright yellow colour. This colour is not sufficiently intense for microscopy, and in any case, some dinitrophenyl derivatives are colourless. The nitro groups were therefore converted to suitably coloured azo dyes by reduction, diazotization, and coupling with a phenol.

D. THE WATER ABOLITION EFFECT

This effect, discovered by Barnard in his investigation of the reactivity of diazonium salts with benzoylated sections, has been found to
operate in the case of FDNB. If a section or smear comes into contact with water before benzylation, blocking by benzylation is complete, and the section will not react with FDNB. The effect was first observed with the diazo salt and with FDNB in preparations which had been dehydrated in absolute ethanol, taken through a series of alcohol concentrations to water and then rehydrated before benzylation. It was later found that exposure to 90% ethanol was sufficient to produce the effect. The phenomenon was interpreted by Barnard and Danielli (1956), in terms of a water-labile protective bond between some cell component and the nuclear groups which react with tetrazotized diaminobenzidine after benzylation. Under anhydrous conditions these bonds are stable and the reacting groups are protected from the benzoic acid, but when the protecting bond is destroyed by water the blocking agent can react. An alternative explanation of the phenomenon would be that the reactive component is leached out by water. The following evidence indicates that this is not the case.

(1) Absolute ethanol → water → absolute ethanol → benzylation → FDNB = unstained nuclei, unstained cytoplasm.
(2) Absolute ethanol → benzylation → water → FDNB = stained nuclei and unstained cytoplasm.
(3) Absolute ethanol → benzylation → water → absolute ethanol → benzylation → FDNB = unstained nuclei and cytoplasm.
(4) Absolute alcohol → benzylation → absolute alcohol → benzylation → FDNB = stained nuclei and unstained cytoplasm.

The result of treatment (1) could be explained by either water labile protective bonds or by the water solubility of the FDNB-reactive component. Treatment (2) shows that the reactive component is not soluble after benzylation. Therefore the absence of a nuclear reaction in (3) (it is present in the control (4)), cannot be accounted for by a water solubility effect. It will be noted that this water effect indicates that under certain conditions benzoic acid is able to penetrate the nucleus and react with the nuclear contents.

The paucity of information on the protection of the nuclear groups from benzylation hazards any theoretical consideration of the mechanism. Little is known of the properties of the proposed bond other than that it is water-labile, and may involve a lipid component. Dehydration after water treatment does not re-form the bond (3 above), and it therefore appears as if the reacting groups and the protecting component are in some specific stereochemical relationship which is destroyed irreversibly by water. It is well known that the availability of the amino acid side chains of a protein is dependent on its physical state, and a number of instances of unreactive tyrosine residues has been reported. Crummer and Neuberger (1943) found that few or none of the tyrosine hydroxyl...
groups of ovalbumin could ionize in the native molecule, but ionized only when the structure of the protein had been destroyed by denaturation. Similarly, Tanford et al., (1955), report that of the six phenolic hydroxy groups of ribonuclease, three ionize instantly and reversibly between pH 9 and 11.5, but the other three slowly, and irreversibly at pH values above 11.5. Thermodynamic considerations suggest that the change in the environment of the latter three tyrosine side chains must be greater than can be accounted for by simple hydrogen bonding. These workers suggest that these three phenolic hydroxyl groups form part of a network of hydrogen bonds which holds the protein molecule together, and the ionization of one of them cannot occur without the simultaneous and irreversible rupture of other bonds. This type of system could account for the behaviour of the protected groups in the nuclei. The protecting bonds are intact during the anhydrous benzoylation, they are disrupted by water, and the liberated amino acid side chains are free to react with the chromogenic reagent.

The identity of the protecting component is not known. Barnard (1961) has found a correlation between the distribution of the protected groups localized by tetrazotized dianisidine and the DNA in the nucleus. Because of the deleterious effect of wax embedding (Section II, B, p. 267), it has been difficult to prepare nuclei optimally fixed for microscopic examination, and the correlation, if any, between the distribution of the groups in nuclei detected by dinitrophenylation after benzoylation, and the distribution of DNA remains uncertain. Positive granules corresponding to heterochromatin have been observed, but the nucleoli are also positive. The possibility of lipid being the protecting component in the nucleus has already been discussed (Section II, B). Goblet cells of the intestine are one of the few cytoplasmic sites that react with FDNB after benzoylation. Barnard has observed that the periodic acid-Schiff's (PAS) method for the detection of 1,2-glycols which was normally blocked by benzoylation, remained positive in these goblet cells after benzoylation, and that this residual reaction showed the water abolition effect. It is reasonable to assume that in this case the protein detected by the FDNB and the carbohydrate protect each other by mutual bonding.

E. The Relationship between the Reaction of FDNB and that of Tetrazotized Dianisidine

It is desirable in any cytochemical investigation to use a number of alternative methods for the study of the same cell component. The reactions of FDNB (Maddy, 1961a, b) and tetrazotized dianisidine (Barnard and Danielli, 1956; Barnard, 1957, 1961) with benzoylated tissue
sections are superficially very similar. Both reagents react after benzylation with cell nuclei and the goblet cells and intracellular mucus of the intestine: the water abolition effect operates in both reactions. The report by Barnard that the cytoplasm of tissues actively synthesizing protein, e.g. pancreas exocrine cells, gives a faint stain with tetrazotized dianisidine after benzylation, has not been confirmed with FDNB. This may be a fundamental difference, or it may be that the amount of protected material in these sites is too small to be detected by FDNB but is detected by the diazonium salt.

A closer investigation has revealed a number of fundamental differences between the components detected by the two methods. The observation that dinitrophenylation after benzylation only slightly diminished the reaction of the diazonium compound, indicated that the bulk of the reactive groups in the nuclei were not identical in the two cases. This inference was confirmed by analysis when it was discovered that the FDNB reacted chiefly with tyrosine residues, and the only detectable reaction of the diazo salt was with histidine side chains. The partial blocking by FDNB of the tetrazonium reaction could be explained by the weak reaction of this reagent with the nuclear histidine. As both reagents react with both tyrosine and histidine side chains, these differences between the two reactions are not easy to understand. The differences may be an expression of the complexities of the nuclear organization, but it is possible that they may be, at least partly, the result of the different reaction conditions and reaction mechanisms of the two reagents. Diazonium salts are known to react as Ar-N⁺ ions (Ingold, 1953) and FDNB as the un-ionized molecule (Bunnett, 1958). A further difference is that the diazonium reaction is not affected by wax-embedding.

The presence of protected groups other than histidine has also been indicated by the report of Barnard (1961) that p-nitrobenzoyl chloride reacts with an unidentified nuclear component after the cells have been blocked by benzyol chloride. This reactive site is distinguished from the reactive histidine by the finding that blocking with a diazonium salt after benzylation does not affect it, and conversely, the histidine component remains reactive towards tetrazotized dianisidine after blocking with p-nitrobenzoyl chloride. The reaction of the p-nitrobenzoyl chloride is not affected by wax-embedding and is not, therefore, identical with the FDNB reaction. This further evidence of the complexity of the nucleus favours the view that the differences between the reaction of FDNB and tetrazotized dianisidine are true manifestations of details of the nuclear organization.

The biological significance of these protected residues (Barnard and Bell (1960) have observed a similar nuclear protection from benzyolation
of groups reactive towards the Sakaguchi reaction) is not understood. The problem is not likely to be solved until the mechanism of the protection and the metabolic activity of the groups are known. At present the suggestion by Barnard and Danielli (1956) that the groups may represent an integral part of the gene, or be active in protein synthesis, cannot be enlarged upon.

III. HISTOLOGICAL PROCEDURE

A. Fixation and Preparation of the Tissues

Alcohol fixation and freeze-drying may be carried out by standard methods. The animal is killed by cervical fracture, thin slices of tissue immediately removed and alcohol fixed in three changes of absolute ethanol, 2 hr in each bath. The fixed material is cleared in methyl benzoate. Tissues are freeze-dried by rapid quenching in a bath of isopentane cooled in liquid nitrogen and dried for 3 days at $-50^\circ$ in an Edwards tissue drier (Bell, 1956). The fixed material is embedded in paraffin wax (m.p. 54$^\circ$), sectioned at 8 $\mu$ and the sections floated-out on ethanol (90% v/v at 47$^\circ$ for 30 sec). Frozen sections of fresh unfixed tissue are cut at 20 $\mu$, rapidly floated-out on isotonic saline and dried in air. The effect of fixation on reactivity is discussed in Section II, B.

B. The Cytochemical Schedules

Note: All reagents are of "Analar" grade unless otherwise specified.

1. Treatment with FDNB

After the investigation of a number of possible variations (Maddy, 1961a), the following procedure based on the method of Danielli, Loveless and Bell (Danielli, 1953) was found to be the most effective.

(a) The wax is removed by two 2-min treatments with xylene.

(b) The slides are washed twice in absolute ethanol. (If freeze-dried material is used it is necessary to complete fixation at this stage by extending the washing periods to 8 min in each bath).

(c) The sections are down-graded to 70% alcohol.

(d) Dinitrophenylation: The medium is prepared by gradually adding, with stirring, a solution of FDNB ($0.4\text{ ml} ; 0.4\text{ g} = 0.28\text{ ml}$) in 39 ml of ethanol to an aqueous NaHCO$_3$ solution (21 ml; 1% w/v). The sections are dinitrophenylated for 2 hr with occasional agitation. (FDNB was obtained from British Drug Houses Ltd. and redistilled before use (Cook and Saunders, 1947).)
(e) Excess reagent is washed off in two 3-min baths of 70% alcohol and the slides taken to water.

(f) The sections are reduced in freshly prepared chromous chloride solution. The chromous chloride is made by boiling a solution of chrome alum (10% w/v) in 0·1 N H2SO4 together with acid washed granulated Zn in a flask fitted with a Bunsen valve. The green product is cooled rapidly. Reduction of 8 μ sections is completed in 10 min; 20 μ sections require 30 min.

(g) Excess reducing reagent is removed by washing three times with water and finally with 0·01 N HCl. The slides are cooled prior to diazotization by carrying out the last water wash and the acid wash (3 min) at 0°-4°.

(h) Diazotization: The sections are diazotized in 0·05 N HNO3 for 5 min. The acid is prepared immediately before use by mixing equal volumes of ice cold 0·1 M NaNO2 and 0·1 N HCl.

(i) The slides are washed twice in water (0°-4°; 2 min and 1 min).

(j) Azo dye formation: The sections are treated with a solution of H acid (8-amino-1-naphthol-3:6-disulphonic acid; 0·1%) or β-naphthol (0·1%) in aqueous Na2CO3 solution (0·5%) for 15 min at 0°-4°.

(k) The slides are rinsed in a solution of Na2CO3 and finally in water.

(l) After dehydration and clearing in xylene, the sections are mounted in neutral Canada Balsam.

2. Benzoylation

The conditions used for benzoylation were those established by Barnard (1961).

(a) The slides are taken to absolute ethanol as described in the above section.

(b) Alcohol is removed prior to benzoylation by a 1-min wash in petroleum ether (80°-100° b.p. fraction) followed by a wash in anhydrous methyl cyanide.

(c) Benzoylation: The sections are benzoylated for 3 hr in a mixture of:

- 50·0 ml methyl cyanide (reagent grade, dried by redistillation over P2O5).
- 4·2 ml benzoyl chloride.
- 2·8 ml pyridine (dried by redistillation over KOH).

Benzoylation is carried out in a Coplin jar over CaCl2 in a desiccator.
(d) Excess reagent is removed in two 3 min alcohol washes.

_N.B._ Care must be taken to see that the reaction is performed under anhydrous conditions.

**IV. ANALYTICAL INVESTIGATION OF THE REACTIVE COMPONENTS IN NUCLEI**

Techniques are not yet available for the unequivocal identification of the chemical components reacting in a single tissue section. This information must therefore be obtained by indirect methods such as treatment of a bulk preparation of tissue by the cytochemical procedure under study, and analysis of the reacted tissue. The reaction of FDNB has been analysed in a bulk preparation of nuclei, and the reactive components in the nuclei identified by the isolation and characterization of their dinitrophenyl derivatives.

**A. Preparation of Nuclei**

Chicken erythrocytes proved to be a suitable source of nuclei for analysis. These cells could be easily obtained in sufficient quantity, their nuclei could be liberated by haemolytic procedures which involved the minimum mechanical damage to the cells, and they represented a homogeneous cell population. The cells were disrupted by freezing and thawing as described by Barnard (1957), and the nuclei collected by differential centrifugation in a medium of isotonic sodium chloride. As the isolation of nuclei in isotonic saline has been reported to produce a phenomenon known as "bleb formation", i.e. the appearance of small bubbles on the surface of the nuclei (Anderson, 1953), the erythrocyte nuclei were frequently examined by phase microscopy during their isolation. No "bleb formation" was observed.

The leaching of protein and ribonucleic acid from the nucleus is, with present methods, an unavoidable disadvantage of isolation in an aqueous medium (Kay _et al._, 1956; Hale, 1957). The removal of these substances can be avoided by resorting to a non-aqueous medium, but these media have their own disadvantages which are accentuated in the present investigation by the anomalous effect of freeze-drying and wax-embedding discovered in the histological work. FDNB reacted with smears of the isolated nuclei, both when used alone and in conjunction with benzoyl chloride, but the possibility of some potentially reactive component being removed during isolation cannot be excluded.
B. Benzylation and Dinitrophenylation of the Isolated Nuclei

The nuclei were fixed by suspension in absolute ethanol, and then treated with benzoyl chloride and FDNB under the same conditions as were used for the histological preparations. Those treated with FDNB without prior benzylation were a bright yellow colour, but no yellow colour was apparent in the nuclei which had also been benzylated.

C. The Fractionation of the Reacted Nuclei into their Constituent Chemical Components

The nuclei were fractionated into lipid, nucleic acid and protein fractions by modification of standard techniques. The lipid was removed by extraction with hot ethanol-ether (3:1 v/v) (Bloor and Sneider, 1934), and the nucleic acid subsequently removed by a 5% (w/v) solution of trichloracetic acid at 90° (Schneider, 1945). The nuclear residue remaining after lipid and nucleic acid removal, the crude nuclear protein, was collected and dried.

Benzylation prior to dinitrophenylation had a profound effect on the behaviour of nuclei during these extractions. The first alcohol-ether extracts of nuclei which had been dinitrophenylated without prior benzylation were bright yellow in colour; the benzylated nuclei, on the other hand, produced a pale yellow extract. In both cases the nuclei were extracted until all the soluble yellow material was removed, and a further extract examined by ultraviolet spectroscopy for colourless dinitrophenyl compounds. No compounds of this type were found. The yellow extracts absorbed strongly in the short ultraviolet region as is characteristic of lipid extracts (Levine and Chargaff, 1952), and also showed an absorption maximum at 360 μm, which was attributed to the dinitrophenyl residues. This absorption peak was strong in the extracts from nuclei which had not been benzylated, but was greatly reduced in preparations from benzylated nuclei. When the extracts were examined chromatographically they were found to contain a small amount of dinitrophenol and a fast-moving yellow component believed to be a dinitrophenylated lipid of the type described by Collins and Wheeldon.

The trichloracetic acid extracts of the nuclei which had not been benzylated were a bright yellow colour, but the extracts of benzylated nuclei showed negligible colour. The ultraviolet spectrum of the yellow extract had two absorption maxima, one at 260 μm and the other at 360 μm, which were attributed to absorption by nucleic acid bases and dinitrophenyl residues respectively. An examination of this extract indicated
that the yellow colour was due to the presence of $\varepsilon$-$N$-dinitrophenyl lysine and 2:4-dinitroaniline. No reliable evidence was, therefore, obtained for a reaction of FDNB with nucleic acids. It is possible, however, that the dinitroaniline was formed by the decomposition of a dinitrophenylated nucleic acid.

D. THE IDENTIFICATION OF THE NUCLEAR COMPONENTS WHICH REACT WITH FDNB

The groups of the nuclear protein which react with FDNB after benzoylation have been identified by hydrolysis of the protein, and characterization of the DNP-amino acids by their chemical properties, their $R_F$ values in paper chromatography, and paper electrophoretic mobilities (Maddy, 1961b). The routine hydrolysis of the protein was carried out in 5·7 $N$-hydrochloric acid, but as DNP-proline were reported to be more stable in 12 $N$ acid, a sample of the protein was hydrolysed in this stronger acid to ensure detection if present (Porter and Sanger, 1948). DNP-amino acids can be separated by ether extraction of the protein hydrolysate into two fractions (Sanger, 1945), an ether soluble fraction containing the non-basic $\alpha$-$N$-amino acids, and the acid soluble fraction containing $\varepsilon$-$N$-DNP-lysine, $S$-DNP-cysteine, im-DNP-histidine, O-DNP-tyrosine, basic $N$-terminal DNP-amino acids, e.g. $\alpha$-$N$-DNP-arginine and dinitroaniline. Bis-DNP-histidine is distributed between the two phases. (The dinitrophenol and dinitroaniline arise as decomposition products). Hydrolysates of nuclear protein which had been benzoylated and dinitrophenylated were fractionated in this way, and the two phases examined by paper chromatography. Three chromatographic solvents were used: (1) tert-amyl alcohol saturated with buffer (0·05 M phosphate; pH 6·0) on Whatman No. 4 paper which had been buffered with the same buffer (modified from Blackburn and Lowther, 1951); (2) 0·05 M phosphate buffer (pH 6·0) (modified from Levy, 1954); (3) butan-1-ol-acetic acid-water (4:1:5). Solvents (1) and (2) were combined for two-way chromatography; the chromatogram was run in the first direction with solvent (2), dried, and the now buffered paper irrigated with tert-amyl alcohol in the second dimension. The only DNP compound in the ether phase from a hydrolysate of protein of nuclei which had been benzoylated and dinitrophenylated, was a small quantity of dinitrophenol. Five nitro compounds were detected in the acid phase: im-DNP-histidine, $S$-DNP-cysteine, $\varepsilon$-$N$-DNP-lysine, O-DNP-tyrosine and dinitroaniline. The colourless histidine, cysteine and tyrosine derivatives were detected by spraying with ninhydrin, Erhlich's reagent ($p$-dimethylaminobenzaldehyde) after reduction with titanous chloride (detects
aromatic nitro groups), and potassium cyanide (detects meta dinitrophenyl derivatives) (Maddy, 1959).

The identification was confirmed by comparison of the mobilities in paper electrophoresis of the unknown compounds with those of known compounds. It was possible to separate the DNP amino acids as their anions in 5·8 n-acetic acid (Maddy, 1961b). The relative rates of movement were in the following order: im-DNP-histidine > ε-N-DNP-lysine > O-DNP-tyrosine > S-DNP-cysteine. The free amino acids had similar mobilities to the im-DNP-histidine and could not be separated from this compound.

The same five compounds were found in hydrolysates of protein from nuclei which had been dinitrophenylated without prior benzoylation, but they were present in much larger amounts. In addition, at least five N-terminal amino acids were detected as their DNP derivatives. Benzoylation, therefore, completely blocks the N-terminal amino acids of the proteins and greatly reduces the reaction of the four protein side chains.

E. THE QUANTITATIVE ANALYSIS OF THE REACTIVE NUCLEAR GROUPS

Quantitative cytochemical methods which are applicable to single tissue sections and smears are now being developed for many reactions, and such a method is desirable for the FDNB reaction. While realizing the utmost importance and the great advantages of a direct analysis of individual tissue sections, considerable information can be obtained by chemical analysis, using standard methods, of bulk preparations of tissue. For this purpose, preparations of erythrocyte nuclei were benzoylated for 30 min, 1 hr, 3 hr and 6 hr, and reacted with FDNB for 3 hr. The nuclei were fractionated and the effect of benzoylation on the dinitrophenylation of the protein determined.

The usual method of determination of α-N-DNP-amino is by direct spectroscopic measurement of the absorption band at 360 mμ, but because of the relatively low extinction coefficients of O-DNP-tyrosine and S-DNP-cysteine, this direct method was not satisfactory for the measurement of the minute quantities of these compounds which were obtained from benzoylated nuclei, and a search was made for a suitable colorimetric method. Reduction, diazotization and coupling of the nitro groups was also not effective. The reduction of the nitro groups by titanous chloride, and the reaction of the amines so produced with p-dimethylaminobenzaldehyde has been developed as a method for the estimation of the compounds (Maddy, 1961b). As it was necessary to measure very small
quantities, the inevitable dilution caused by elution was avoided by determining the DNP-amino acids directly on the paper after separation by paper electrophoresis. The nitro groups were reduced by spraying the paper with titanic chloride solution and warming, and the coloured Schiff's bases produced by spraying the amines with the Ehrlich's reagent and drying under standard conditions. The colour was measured in a densitometer, and a linear relationship was obtained between con-

![Fig. 2. The effect of benzoylation on the subsequent dinitrophenylation of the tyrosine side chains of the nuclear protein obtained from chicken erythrocytes.](image)

centration and absorption. Operational and hydrolytic losses were inevitable during this procedure and recovery factors were determined for each compound.

By this method the amount of $\varepsilon$-N-DNP-lysine and O-DNP-tyrosine in the nuclear protein erythrocytes which had been dinitrophenylated without prior benzoylation was determined, but the extent of the substitution of the cysteine and histidine side chains was too small to be measured. Benzoylation was found to reduce the lysine derivative to unmeasurable quantities, but the tyrosine derivative, although greatly reduced, could still be estimated. The effect of benzoylation on the dinitrophenylation of tyrosine is shown in Fig. 2, where it will be seen that after 3 hr benzoylation only 2% of the total nuclear tyrosine available to FDNB reacts and that no further benzoylation was detected between 3 and 6 hr.
V. CRITIQUE OF THE METHOD

The dinitrophenylation of sections which have not been benzoylated has never presented any difficulty to the present author or other members of Danielli's group, but Pearse (1953) and Burstone (1955) have both criticized the method. The poor reaction reported by Pearse may be a result of the low temperature (0°-4°) at which he allows the reaction to take place. (Proteins are usually reacted with FDNB at room temperature). The observations of Burstone are more difficult to understand, for while he found reaction of FDNB in the presence of bicarbonate and reduction of the nitro groups with chromous chloride ineffective, prolonged reaction (20-24 hr) with FDNB containing sodium hydroxide and reduction with sodium hydrosulphite at 45° was successful. Burstone states: “These (chromous and stannous chlorides) are relatively weak reducing agents, and it was only with more vigorous reduction with sodium hydrosulphite that good results were obtained.” This cannot be regarded as a very likely explanation.

Pearse discussed the structures resistant to benzoylation as localized by diazonium salts, but not by FDNB. He reported this resistance to benzoylation to be partly due to mild heating, and although this finding has not been confirmed by Maddy with FDNB, or by Barnard with tetrazotized dianisidine, the precaution was taken of carefully controlling the temperature during the mounting of wax sections.

The work of Maddy supports Burstone’s report of the reaction of FDNB with cytoplasm, elastic cartilage, and collagen, but not his claim that nuclei and mucous cells are negative. These negative reactions might have been due to a leaching out of reactive substances similar to that described for Sakaguchi positive material by Barnard and Bell (1960). In an account of the reaction of tetrazotized dianisidine with tissue sections, this same author reports pre-treatment with FDNB to increase the reaction of the tetrazonium salt. This report is very difficult to understand and is contrary to the findings of both Barnard and Maddy who find that, as would be expected, pre-treatment with FDNB reduces the reaction of tetrazonium salts.

A major criticism of the quantitative analysis of the isolated nuclei is the possibility, already mentioned, of a reactive component being lost during isolation. This source of error will best be investigated when a microspectrophotometric technique applicable to blood smears is available.

VI. RESULTS TO DATE

The reaction has been studied in a wide range of rat and chicken tissues (Table I). The results are illustrated in Figs. 3–5. FDNB without
Fig. 3. Air dried smears of chicken erythrocytes. Magnification: × 600. A: Dinitrophenylated without prior benzoylation. Both nucleus and cytoplasm are stained. B: Dinitrophenylated after benzoylation. The stain is confined to the nucleus.
Fig. 4. Frozen section of rat liver. Dinitrophenylated after benzoylation. The stain is confined to the nuclei. Magnification: × 500.

Fig. 5. Frozen section of chick kidney. Dinitrophenylated after benzoylation. The stain is confined to the nuclei. Magnification: × 500.
Fig. 6. Freeze-dried section of rat intestine. Dinitrophenylated after benzoylation. Note positive goblet cells and nuclei.
prior benzyolation of the tissues gave an intense overall stain in all the specimens examined (Fig. 3 (A)). After benzoylation of the tissues, staining was generally confined to the nuclei (Figs. 3 (B), 4, 5, 6). Cytoplasmic staining after benzoylation was restricted to a few characteristic sites, the goblet cells of the intestine, the intracellular mucus of the stomach epithelium, and a submucosal band of the stomach and intestine. Microscopic examination of the preparations indicated that benzoylation for 1 hr produced the complete effect, i.e. the nuclei were stained and the cytoplasm was completely unstained. Extension on the time of benzoylation to 12 hr did not apparently reduce the nuclear staining. After benzoylation for 30 min there was a very faint stain in the cytoplasm, and the nuclei were slightly more intense than those which had been benzoylated for 1 hr. Although it appeared from these experiments that the full benzoylation effect was produced after 1 hr, quantitative analyses later showed that 3 hr benzoylation was necessary to produce the full effect (Maddy, 1961b).

The effects of fixation and wax-embedding have already been discussed (Section I, B). The results of the analytical investigation of the reaction are included in the account of that work (Section III).

ACKNOWLEDGEMENTS

The author is indebted to Professor J. F. Danielli, F.R.S., for suggesting the problem, and for his constant advice and encouragement. Most of the work was carried out during the tenure of a Medical Research Council Scholarship at King’s College, London.

REFERENCES


