I. INTRODUCTION

It is useful in protein cytochemistry to distinguish between methods providing information (a) on different protein species defined by their...
activity (at present, this must be enzymic or antigenic or, possibly, metabolic) and (b) on different protein side-chain groups. Of the methods available for this latter purpose, the most promising would be the use of reagents selective for particular chemical groups. Such reagents must form stable covalent bonds at specified sites, and meet various other, often rather stringent, requirements for adequacy (cf. Danielli, 1953; Barnard, 1958).

These latter reagents may usefully be chromogenic (i.e. introduce a selective absorption of light, either visible or ultraviolet, at the acceptor site) or, for other techniques, fluorescent or isotopic or electron-scattering. Only the chromogenic case has so far been developed to any significant extent. Due to the overlapping reactivities of different protein groups, sequences using non-chromogenic blocking agents may be valuable for narrowing the range of reaction of a chromogenic reagent, as proposed by Danielli (1950). In the present report, some investigations of acylation and diazonium coupling as such blocking and chromogenic methods, will be discussed.

In methods of this type, it is hazardous to rely entirely on predictions from simple organic chemical considerations as to which groups will react with particular reagents. Knowledge and interpretation of the reactivities of groups in proteins in simple solution is itself, at present, at a rather unsophisticated level, while complexities are introduced by the molecular combinations and physico-chemical conditions present in tissue specimens. In addition to the reactivity factors, steric factors are often of great importance here, as in the difficult problem of the availability of particular protein groups in various conditions.

In the face of these uncertainties, the best approach in interpreting any particular case would appear to be, after taking into account such relevant information on protein behaviour as is available, to determine by micro-analytical methods on reacted material which groups have actually reacted. This empirical approach, in which the stages of deduction and cytochemical analysis are followed by a biochemical validation, is illustrated in the case of the method described here.

II. THEORY OF THE METHODS

A. PREDICTED REACTIVITIES

1. Diazonium Coupling in Cytochemistry

The familiar coupling reaction of diazonium compounds

\[
\text{Ar.N}_2\text{OH}^- + \text{R.H} \rightarrow \text{Ar.N=N.R} + \text{H}_2\text{O}
\]

is of potential value in cytochemistry since it occurs relatively rapidly in
aqueous solution at slightly alkaline pH and at 5°, to yield coloured azo compounds.

When a diazonium compound is applied thus to a tissue specimen, the following may react:

(i) Certain groups in protein side-chains (to be discussed in detail below).

(ii) Some aliphatic —NH₂ and —NH— groups, to form triazenes \((\text{Ar.} \text{N} = \text{N.} \text{NH.} \text{R})\). These will in practice generally occur in proteins (see below).

(iii) Naturally occurring phenols and amines, e.g. adrenaline, 5-hydroxy-tryptamine, oestrogens, etc. These will normally be removed in the pre-treatment of the tissue specimen. In exceptional cases such a low molecular weight compound might become attached in some way to a macro-molecular structure. A reaction from this source may occur in the enterochromaffin cells of the alimentary tract.

(iv) Other potential couplers do not, so far as is known, occur naturally. An important group of possible exceptions includes the purine and pyrimidine bases of polynucleotides. Some free pyrimidines do in fact couple (Burian, 1907; Johnson and Clapp, 1908; Fischer, 1909) but in an abnormal variant of the reaction occurring only in strong NaOH solution. Thymine reacts with diazobenzene sulphonic acid (Hunter, 1936), but only in the presence of hydroxylamine and strong NaOH. Even so, nucleotides do not appear to react at all in these cases (Burian, 1907; Fischer, 1909). Nucleic acids in the isolated state do not appear to react with diazo compounds at pH 9 (Gomori, 1952; Stuart-Webb, unpublished observations in this laboratory). The slight possibility remains, of course, that some structural feature in the \textit{in situ} state introduces the capacity for coupling at moderate pH.

(v) Diazonium coupling is frequently applied to form a colour at sites where a reactive acceptor group, e.g. a naphthol, has been artificially introduced by enzyme action or by a prior reaction method (e.g. at SH or aldehyde groups). The occurrence of case (i) must then be borne in mind in designing sequences and in spectrophotometry.

Case (i), which can normally be expected to predominate, must be considered in more detail. Unambiguous, detailed analyses of the diazo coupling behaviour of proteins themselves have not yet been reported, most studies being on model compounds.

Free histidine and tyrosine were shown to couple in the classical investigations of Pauly (1904, 1915), and it has since been confirmed that imidazoles, including histidine derivatives, form stable, true C-azo dyes (Fargher and Pyman, 1919; Pyman and Timmis, 1922; Diemair and Fox, 1938). Tyrosine residues in synthetic polypeptides couple as expected in
the 3-position (Sela and Katchalski, 1955). The spectra of azo-proteins support mono-coupling at tyrosine and histidine residues (Gelewitz et al., 1954).

Triazenes may be formed at amino groups in proteins (Fraser and Higgins, 1953). Diazo compounds can react with free amino acids to deaminate them (Zahn et al., 1954; Howard and Wild, 1957) or to form a triazene which may unexpectedly be stable to dilute HCl (Busch et al., 1934). ε-Aminocapric acid, a model for a lysine residue, forms a bis-triazene (Howard and Wild, 1957). Thus, the coupling reaction of α- and ε-NH₂ groups in a given protein is uncertain. If triazenes are formed, and if they should remain after dilute acid treatment, they will nevertheless introduce relatively little absorption of visible light. It has also been claimed that protein arginine groups will form triazenes (Howard and Wild, 1957), but this is on the basis only of the reaction of methylguanidine. There is no direct evidence yet that these highly basic groups are affected in proteins.

Simple thiols (Duffin and Kendall, 1954; Howard and Wild, 1957) and cysteine (Zahn et al., 1954) react with diazo compounds, but the products vary greatly in stability.

Tryptophane coupling has previously been the subject of contradictory and inconclusive reports. It has recently been found, however, that free tryptophane couples with diazobenzene sulphonic acid to a slight extent, but in N-acetyl tryptophane and gramicidin to a considerable extent (Barnard, 1959). These stable dyes are red in acid and pale yellow-orange in alkali.

In summary, a diazonium reagent might conceivably modify any one of the more reactive protein side-chain groups, and it would be unsafe to exclude this possibility in any given protein case without further evidence. However, reaction to form strongly-coloured products (observed at alkaline or neutral pH) can be expected only in the cases of tyrosine and histidine, and to a smaller extent tryptophane, residues.

2. Acylation

Acylating agents, e.g. benzoyl chloride [PhCOCl] and acetic anhydride [(CH₃CO)₂O], are capable of reacting at sites (e.g. —NH₂, —OH, —SH and others) in a large number of cellular components, including proteins, lipids, polysaccharides and nucleic acids. The multiplicity of these reactions reduces the usefulness of these reagents for cytochemical localizations, but they are valuable as blocking reagents. Chemically, acylation is usually performed in aqueous alkali, but the alternative method with an anhydrous medium containing an organic base has been found to be milder and more efficient in cytochemical applications. In
particular, dry acetonitrile with one equivalent of pyridine has been found here to be a very satisfactory vehicle. When applied thus in protein cytochemistry, acylating reagents should react at $-\text{NH}_2$ (lysine and N-terminal), phenolic (tyrosine), $-\text{OH}$ (serine and threonine) and $-\text{SH}$ (cysteine) groups. In addition, some arginine side-chains may react (see Section D, 3 below).

The stability of these products will vary. The N-acyl compounds will normally be of high stability, requiring heating in acid for their hydrolysis. Protein S-acyl derivatives can in general be expected to be of lower stability, and should be split readily in aqueous alkali at room temperature (cf. Neuberger, 1938; Fraenkel-Conrat, 1944). Their ease of hydrolysis is probably markedly dependent on their precise molecular environment; migration of the acyl radical to neighbouring amino groups in slightly alkaline solution may also occur in some cases (Wieland et al., 1953). The O-acetyl groups can be expected to undergo hydrolysis at significant rates at about pH 10 and above at room temperature (cf. Herriott, 1935; Olcott and Fraenkel-Conrat, 1947; Ram and Maurer, 1958), although it is possible that some might be split at a lower pH.

The important case of the reactivity of the imidazole ring of histidine is slightly more complicated. In aqueous alkali, reaction with benzoyl chloride gives a stable product in which the imidazole ring is opened, while in anhydrous medium normally an N-acyl imidazole is formed, which decomposes in water leaving the imidazole ring unchanged (for a fuller account and literature references, see Barnard and Stein, 1958). However, in the present studies it has been found that by reacting with anhydrous benzoyl chloride in the acetonitrile-pyridine medium, protein histidine groups in a large number of sites can be fully and stably blocked. It is not yet clear whether this is so because a type of ring-opening reaction occurs in this medium, or (less probably) because a stable N-acyl imidazole derivative is formed in the proteins.

Those groups in proteins which can react with diazonium hydroxides lose this capacity when they are acylated (since their nucleophilic character is then in all cases lost). Hence, in principle, effective acylation should block the diazonium coupling of proteins.

It is clear, however, that firm, general predictions cannot be made about the reaction with acylating agents of the protein groups concerned, nor about the stability of the products. In addition, various acylating agents, applied cytochemically, show differing reactivities (see Section D, 4 below). Hence, analysis is required of each particular case for accurate interpretation of the observed results of cytochemical acylations.
B. The Cytochemistry of the Benzoylation-Diazonium Method

1. Applications to Cytochemistry

Application of the diazonium coupling reaction to cytochemistry was made quite early (Clara and Canal, 1932; Lison, 1936). In these early methods the method was regarded as showing phenolic groups only, and the colours obtained were generally orange or yellow. A more satisfactory procedure is that used by Danielli (1947) in which tetrazotized benzidine is used. This forms an azo link at one end of the molecule with, say, a tyrosine group in the protein. After suitable washing, the second, free diazo group is coupled with a naphthol, giving a strong red-purple colour due to the bis-azo dye:

\[
\text{Protein} - \text{OH} \rightarrow \text{Protein} - \text{OH}
\]

\[
N=N-\text{Protein} - \text{OH} + \beta\text{-naphthol} \rightarrow \text{Protein} - \text{OH}
\]

(1)

No dye is applied to the tissue; excess reagent can be washed away at each stage and the naphthol component can be varied as a check on adsorption artefacts. This method, referred to as tetrazonium coupling, has been used in most of the work to be discussed here.

The blocking of diazonium coupling by benzoyl chloride was first applied cytochemically in the pioneering attempt of Mitchell (1942), who at that time proposed it as a method for nucleotides. He employed the normal chemical method using benzoyl chloride in 2 N NaOH solution (6 to 12 hr), followed by coupling using diazotized sulphanilic acid. The alkali treatment is drastic and the results were patchy; the reaction was generally negative in normal cells (though a red stain was noted in the cytoplasm of some irradiated cells). Mitchell correctly suggested, however, that tyrosine and histidine groups give rise to an overall coupling reaction, and that this should be blocked by benzoyl chloride.

The use of benzoyl chloride in pyridine (12 hr) was proposed by Danielli (1950). This is clearly a much less drastic method. He has shown that a decrease is thus produced in the coupling ability of the bands in *Drosophila* salivary gland chromosomes.

In the investigations to be discussed here, 10% benzoyl chloride in acetonitrile containing one equivalent of pyridine, applied at room temperature for 3 hr, was normally used and found to be highly efficient.
Acetonitrile is readily dried (as is required, see below) and has solvent properties very similar to those of its analogue, ethanol, to which, in use, the tissue has already been exposed; it seems less likely to damage the tissue on long exposures than some other possible solvents.

2. Total Coupling Reaction

A diazonium reagent applied to a tissue section produces a strong colour reaction throughout all the cells. This, of course, simply indicates the general distribution of proteins. It might be possible to apply this reaction for the micro-spectrophotometric estimation of total protein, but only in an approximate manner, since the content of tyrosine and histidine groups varies between protein types, as does the availability of those groups when present. The method would, less imprecisely, demonstrate the sum of available tyrosine and histidine, but even for this purpose there would be complications due to the possible contribution at some sites of tryptophyl and other groups, and to variations in the ratio of tyrosine to histidine (since the extinction coefficients of their products differ). Spectral studies might lead to a modification whereby the contributions of different groups could be distinguished.

3. Effect of the Blocking Reaction

When the diazonium reaction is preceded by anhydrous benzoylation, the coupling colour is, in general, no longer developed in the cell cytoplasm. In the nuclei of all cells (and in one or two specific cytoplasmic sites), however, a strong and characteristic coupling reaction persists. This is not affected even by prolonged benzoylation, and is the characteristic BDC reaction. (To avoid cumbersome repetition, this abbreviation will be used here to denote the use of benzoylation and diazonium coupling in sequence. Included here is the use of a tetrazonium reagent as a special case of diazonium coupling at a tissue group).

The efficiency of the benzoylation method cannot be in doubt since renewed application of the reagent gives no change in the result, and the medium after use will at once react with water, aniline or phenol; in any case, the benzoylation is quite effective on the cytoplasmic proteins.

Thus, cytoplasmic proteins behave as predicted, but some component in the cell nucleus shows an unexpected resistance to benzoyl chloride, but not to diazonium coupling. This component has been identified (Barnard and Danielli, 1956) by reaction of tissue in bulk under conditions similar to those used cytochemically, followed by fractionation and analysis (see Section IV, C, for the procedures used). The component concerned has been shown to consist of protein histidine residues in a nucleoprotein fraction of the nucleus.
4. The Water Effect

During the examination of this reaction, a remarkable effect of water on the reactivity was discovered. The reaction is normally obtained in a frozen-dried tissue specimen, fixed in alcohol. Exposure of such a specimen to water for only one minute, prior to benzoylation and coupling, abolishes the colour reaction. On the other hand, specimens can remain in water for long periods after benzoylation without apparent effect on the coupling. The abolition is independent of the pH of the aqueous medium, and is obtained even after 1 min in 70% alcohol. Short exposures to still higher alcohol concentrations weaken the reaction. Water abolishes the subsequent reaction in all types of cell investigated; at the most, a trace of exceptionally faint, diffuse staining is obtained.

This effect is not due to the removal of the chromogenic component in the water, as shown by the results of the following sequences (all on material frozen-dried and fixed in alcohol):

1. Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Water $\rightarrow$ Tetrazonium coupling.
2. Water $\rightarrow$ Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Water $\rightarrow$ Tetrazonium coupling.
3. Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Water $\rightarrow$ Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Water $\rightarrow$ Tetrazonium coupling.
4. Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Alcohol $\rightarrow$ Benzoylation $\rightarrow$ Water $\rightarrow$ Tetrazonium coupling.

The nuclei stain in (1) and (4), but not in (2) and (3).

For any interpretation in terms of the removal of the chromogenic component in water, sequence (3) shows this removal must be assumed to occur in water after benzoylation as well as before it, whereas a longer water exposure occurs after benzoylation routinely, (1), with positive results. A lengthy renewed dehydration in alcohol after the water exposure, and before benzoylation, does not restore the reactivity.

This effect of small amounts of water explains the irreproducibility found in the reaction before fully anhydrous conditions were applied, and perhaps why failure to repeat it has occasionally been reported by other authors (e.g. Gomori, 1952; Burstone, 1955). Moisture must be avoided at all stages until after benzoylation.

5. Interpretation of the Reaction

The reaction has been interpreted (Barnard and Danielli, 1956; Barnard, 1960a) as showing protective bonds at histidine groups in the nucleoprotein. These bonds, having properties similar to fairly strong hydrogen bonds, protect the imidazole ring of these histidine residues from benzoylation while elsewhere histidine (and other) groups react. In water, these labile bonds are split, and coupling can occur. Similarly,
after water, benzoylation can occur. The bonds apparently cannot reform in alcohol after a water treatment. The relation to other similar components, and to the *in vivo* state, are discussed in Section VI.

![Chemical structures](image)

**FIG. 1.** Dyes produced by tetrazonium coupling (using dianisidine and H acid) at a histidine group in the protein chain (I), and at N-benzoyl histidine (II). (The assignment of the coupling to the 2-position in the imidazole ring, as shown, is not established.)

### C. **Spectral Characteristics of the Reaction Product**

The two chromophoric azo groups and the conjugated aromatic system present in the molecule of the reaction product I (Fig. 1) will give rise to a high absorption peak towards the red end of the visible spectrum. The colour actually obtained in the cytochemical reaction varies with the

<table>
<thead>
<tr>
<th>Naphthol</th>
<th>Synonym</th>
<th>Final colour of reaction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-naphthol</td>
<td>—</td>
<td>Deep red</td>
</tr>
<tr>
<td>2-naphthol-3:6-disulphonic acid</td>
<td>R acid</td>
<td>Red-brown</td>
</tr>
<tr>
<td>1-amino-8-naphthol-4:6-disulphonic acid</td>
<td>K acid</td>
<td>Purple (slightly brown)</td>
</tr>
<tr>
<td>1-amino-8-naphthol-3:6-disulphonic acid</td>
<td>H acid</td>
<td>Dark purple-violet</td>
</tr>
<tr>
<td>1:8-dihydroxynaphthalene-3:6-disulphonic acid</td>
<td>Chromotropic acid</td>
<td>Golden brown</td>
</tr>
<tr>
<td>8-hydroxyquinoline</td>
<td>Oxine</td>
<td>Red</td>
</tr>
</tbody>
</table>
naphthol component used (Table I). The absorption curve of the BDC reaction product in nuclei has not yet been determined. A model for this product is the compound II, obtained by coupling with $\alpha$-N-benzoyl histidine. The latter is used in preference to free histidine, to mimic a peptide, and to avoid the presence of the free amino group which has been found here in practice to react immediately with tetrazotized dianisidine.

The preparation of compounds of type II in a pure state, for use in spectroscopic calibrations of the methods, is not altogether simple. When N-benzoyl histidine is reacted in free solution with one equivalent of tetrazotized dianisidine, followed later by H acid, several by-products are also formed. These must be separated chromatographically, to follow the course of the reaction and to determine the true spectrum of II (Fig. 2). No evidence for coupling occurring twice in the imidazole ring has so far been found in this particular system by paper chromatographic analysis, although such bis-coupling has been observed in similar histidine derivatives with some simple diazonium compounds.

The spectrum of the dye product II is found to contain a high peak in the region of 600 m$\mu$ in alkaline or pyridine solution. A quite similar spectrum is found with other related bis-azo dyes derived from dianisid-
dine. It would appear, therefore, that micro-spectrophotometric determinations of the absorption curves on cells after such tetrazonium reactions are unlikely to give direct information on the nature of the sites of attachment. Such discriminations might be possible in some cases if suitable diazonium compounds were employed, but the micro-spectrophotometry would be difficult to accomplish with the sensitivity then required.

Solutions of these bis-azo dyes obey Beer's Law fairly well over the concentration ranges that can conveniently be examined (Table II).

### TABLE II

**Variation of Absorbance of Solutions of Dye Compound II with Concentration and Path Length**

<table>
<thead>
<tr>
<th>Relative concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Path length (cm)</th>
<th>Absorbance (580 μ)</th>
<th>% Theoretical absorbance&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>0.224</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>0.440</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>0.426</td>
<td>98</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>0.403</td>
<td>91</td>
</tr>
<tr>
<td>40</td>
<td>0.5</td>
<td>0.986</td>
<td>90</td>
</tr>
<tr>
<td>200</td>
<td>0.1</td>
<td>0.917</td>
<td>84</td>
</tr>
<tr>
<td>400</td>
<td>0.1</td>
<td>1.82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83</td>
</tr>
</tbody>
</table>

<sup>a</sup> Taking lowest concentration employed as 1. The highest concentration is roughly $4.6 \times 10^{-4}$ M (measurements in Hilger Uvispek spectrophotometer; pH 10).

<sup>b</sup> Relative to absorbance of most dilute solution. Theoretical value is that assuming Beer-Lambert Law holds.

<sup>c</sup> May be inaccurate due to high absorbance.

**D. Relation to Other Cytochemical Methods**

1. **DNA**

A relationship to the Feulgen reaction (for DNA) has been recognized from the start of work on the BDC reaction. The intra-nuclear distribution of the two stains is similar in all types of cell examined. For a detailed comparison, the large nuclei of active pancreatic acinar cells may be taken. It has been found possible to develop the BDC stain and the Feulgen stain separately in sequence in the same nucleus. Thus, after BDC reaction and photography, reduction (by titanous chloride) splits off the azo compound leaving the nucleus colourless and unchanged; the Feulgen sequence is then performed, followed by re-photography. The
two stains in any given nucleus are found to be superimposable, as illustrated in Fig. 3.

To discover whether the BDC reaction can occur after the removal of DNA from the nucleus is rather difficult. Removal prior to benzoylation, e.g. by desoxribonuclease (DNase), would involve an aqueous medium and therefore automatic abolition of the reactivity. Removal by DNase after benzoylation might be affected by the benzoyl groups present, but in any case it would be difficult to interpret the subsequent reaction, since newly available groups in the protein may be revealed in structural alterations involved in the removal of DNA from the nucleoprotein complex.

Alternatively, the effect of removal of the purine and pyrimidine bases of the DNA can be studied. Again, the complication of changes in the availability of protein groups is present to some extent, and the results can provide only a very approximate comparison with the normal BDC reaction.

The Feulgen-type hydrolysis (N HCl at 60°) removes initially mainly purines only, and later mainly pyrimidines and other parts of the DNA molecule (Ely and Ross, 1949; Woods, 1957; Walker and Richards, 1957), this leading to the well-known bell-shaped curve for the variation of Feulgen intensity with hydrolysis time. After benzoylation, followed by hydrolysis, the Feulgen staining reaction is apparently unaltered, but the rate of decrease in intensity after the optimal hydrolysis time appears (by eye) to be noticeably slower, presumably due to introduced benzoyl groups. Hydrolysis after benzoylation (in rat pancreas and intestine and fowl erythrocytes) appears to leave the subsequent coupling reaction almost unimpaired up to a 20–30 min hydrolysis period. At this point the Feulgen reaction (after benzoylation) is very weak, and it can be presumed that the majority of the purines and pyrimidines have been removed. Even after 60 min hydrolysis, when the Feulgen reaction is abolished, some coupling reaction can be obtained (Fig. 4). It seems probable that the basic proteins, which become highly benzoylated, are insoluble in the N HCl. The total coupling reaction in nuclei in parallel unbenzoylated sections is considerably decreased after similar hydrolysis. There is also, in benzoylated material, a slight cytoplasmic coupling after 60 min hydrolysis, probably indicating the removal of a small number of blocking benzoyl groups.

Due to the complications mentioned, only a broad qualitative assessment can usefully be made, in these over-hydrolysis studies, but they were of value in indicating that after benzoylation the DNA bases and the coupling component can be distinguished and separated. The question of some initial association between these components in the intact nucleus remains, of course, unaffected.
Fig. 3. Rat pancreas. Nucleus, with prominent nucleoli. A: BDC reaction. B: the same nucleus re-photographed, after reductive removal of tetrazo stain followed by the Feulgen reaction. Magnification: $\times 3200$. 

(A) (B)
Fig. 4. Rat intestine. Benzoylated, followed by hydrolysis in N-HCl at 60°, 60 min., then tetrazonium coupling. Magnification: × 480.
2. Carbohydrate Groups

The BDC reaction is found to be positive, and often intense, at certain non-nuclear sites which are known to be characterized by the presence of muco-proteins, e.g. intestinal goblet cells, cartilage, etc. (see Section VI, A). Some light is thrown on these exceptions by studies (Barnard, 1959) on the effect of the same benzoylation treatment on the periodic-acid-Schiff (PAS) reaction at these sites.

The PAS method (Hotchkiss, 1948) reveals the distribution of non-diffusible carbohydrate containing the 1:2 glycol group. Carbohydrate hydroxyls of this type normally react readily with benzoyl chloride, and the products no longer give an aldehyde with the periodate oxidation treatment (cf. Gersh, 1949); hence benzoylation should block the PAS reaction. In practice, when frozen-dried cells are subjected to anhydrous benzoylation followed by the PAS reaction, two types of behaviour are observed:

(i) A large number of characteristic PAS-positive sites lose the capacity for this reaction after benzoylation; usually 30–60 min suffices.

(ii) In certain other sites, which include intra-cellular mucus in the duodenum, colon, stomach, and some sites in salivary glands and in cartilage, the PAS reaction cannot be blocked thus, even with very prolonged benzoylation times. But a prior brief exposure to water (or aqueous alcohol) allows benzoyl chloride to block the PAS reaction there. This second group also, in all cases, gives the BDC reaction. Further, both reactions show the same sensitivity to prior water treatment, with both disappearing at the same point. It was shown (Barnard, 1959) by interferometric measurements that this effect of water cannot be ascribed to the loss of material, to any significant extent, from the cells.

Hence it seems likely that at these latter sites in the frozen-dried cell, carbohydrate hydroxyls are bonded on to protein. It is assumed that histidine groups are involved although no analysis has been made here; it is not unlikely that other protein groups, not detectable by the coupling reaction, are also involved. The complex remains intact in anhydrous conditions, and these two components are thus mutually protected from benzoylation. But in a more polar medium the combination is irreversibly disrupted; hence the histidine reacts with a diazo compound in aqueous buffer, and the glycol reacts with periodate in aqueous alcohol, or both components react with benzoyl chloride after water treatment.

What significance this carbohydrate-protein combination has in the intact tissue is unknown. For mucus, the newly synthesized or native form appears to be required. In cartilage, interesting discriminations between different stages of cellular development have been observed by this reaction.
3. Arginine Groups

The anhydrous benzoylation treatment appears also to block cytoplasmic arginine groups, while many nuclear arginine groups are not so blocked and can then be revealed and measured by the Sakaguchi reaction in alkaline medium (Barnard and Bell, 1960). These latter groups are believed to be those associated with DNA in the nucleoprotein.

These constitute, then, a further set of protein groups differentially protected from benzoyl chloride. The involvement of these arginine groups in bonding in the nucleoprotein clearly differs from that of the protected histidine groups: the arginine reaction is not affected by water (or alkali) pre-treatment, and is obtained equally after freeze-drying, freeze-substitution and several chemical fixatives. A relatively stable, salt-linked structure is probably concerned.

The versatility of the anhydrous benzoylation treatment is thus further demonstrated, in that the guanidino group, in spite of its high affinity for protons, is nevertheless acylated in this medium when not held in the charged form by neighbouring phosphate groups.

4. Reaction with other Blocking Agents

a. Acetylation

Anhydrous acetylation, like benzoylation, should block tyrosine groups to form phenolic esters, normally stable at room temperature up to about pH 10 (see p. 207). With protein histidine residues, whether reaction will be observed is less certain. The product might be either the N-acetyl imidazole derivative, highly unstable to water (cf. Barnard and Stein, 1958) or a stable acetylated product in which the imidazole ring has been destroyed. The latter reaction is well known in the reactions of benzoyl chloride with certain histidine derivatives (see p. 207), but does not appear to have been established in the case of acetic anhydride. However, it occurs similarly with iso-valeryl chloride in aqueous alkali (Windaus et al., 1921) and with the acetylating agent ketene on imidazole in dry, cold ether (Neuberger, 1938). The possibility that it may occur in the reaction of proteins with acetic anhydride cannot, therefore, be dismissed.

With benzoyl chloride, tetrazo (and diazo) coupling is generally blocked in the cytoplasm, and after water treatment in the nucleus; it follows that the product of benzoylation at both tyrosine and histidine residues in cellular proteins must be stable under the conditions of the first coupling reaction (pH 9, 4°, about 20 min). It is therefore of interest that acetic anhydride, applied in the same acetonitrile-pyridine medium, does not block coupling to the same extent. Coupling after acetylation gives a positive reaction in both cytoplasm and nucleus. The cytoplasmic
reaction is decreased, however. This may be only a kinetic difference, but if so it is considerable. Acetic anhydride (3 M, 6 hr or 1 M, 18 hr) leaves unblocked (at room temperature) many sites that are blocked by benzoyl chloride (0.7 M, 2 hr). Still longer exposures or elevated temperatures (cf. Pearse, 1953) might therefore be required, but would be undesirable. Alternatively, it might be that some protein histidine groups are not effectively blocked by acetic anhydride, and are thus demonstrated. Prior water treatment does not noticeably increase the blocking obtained, and the sequence

Benzoylation→Water→Acetylation→Tetrazo coupling

which presents an opportunity for the acetic anhydride to react at the shielded histidine groups in the nucleus, still produces a positive nuclear reaction (although it is not known whether this is quantitatively as great as the normal BDC stain). Recently, the rate and the extent of the total reaction of acetic anhydride at cellular sites have been measured absolutely in one cell type, using an isotopic cytochemical method (Barnard and Marbrook, 1961).

The acetylation treatment can be as effective as benzoylation in blocking the PAS reaction, although a true kinetic difference is then observed. At a number of sites, with 2·7 M acetic anhydride 1–2 hr is required to obtain the blocking produced by 0·7 M benzoyl chloride in \( \frac{1}{2} \)–1 hr.

b. *p*-Nitrobenzoyl Chloride

This reagent has been employed in these studies (i) as a blocking agent, and (ii) as a chromogenic reagent. In the latter case, the colour is developed by a procedure following the general method proposed by Danielli (1950, 1953) for the use of nitro compounds as chromogenic reagents.

*p*-Nitrobenzoyl chloride (2 g) is applied in dry acetonitrile (50 ml) containing pyridine (3·2 ml), for 2 hr, followed by washes in acetonitrile, hydration and the colour development procedure (reduction with chromous chloride, 15 min, diazotization and coupling with H acid; details are described by Maddy, 1961). Applied thus, the reagent is of value in indicating the sites and extent of reaction of benzoyl chloride. It produces an intense red-purple colour throughout the nucleus and cytoplasm of all cells, with the nuclei staining particularly intensely.

This reaction should give the sum of all groups available and reactive towards an acylating agent. The reaction is not confined to proteins. Thus, it is noticeable that plant cell walls react strongly due to reaction at carbohydrate hydroxyl groups; similarly, extra-cellular mucus and basement membranes also react strongly.
However, it cannot be assumed that the cytochemical reaction of 
$p$-nitrobenzoyl chloride is identical with that of benzoyl chloride and, 
indeed, this has now been shown not to be the case. It is of interest, as a 
case of differential reactivity, to examine the comparative cytochemical 
properties of these two reagents:

(i) $p$-Nitrobenzoyl chloride, like benzoyl chloride, blocks tetra-
zonium coupling in the cytoplasm, but not in the nucleus. Similarly, 
brief prior exposure to water prevents this nuclear reaction.

(ii) When benzylation is followed by $p$-nitrobenzoyl chloride 
used chromogenically, a colour reaction persists in the nuclei only, 
with a distribution very similar to that of the BDC reaction. Brief 
prior exposure to moisture abolishes this reaction too.

(iii) The same cytoplasmic sites (e.g. goblet cells, pancreatic 
acinar basal cytoplasm) that, exceptionally, give the BDC reaction, 
are positive also in reaction (ii) here, and a similar water effect is 
shown.

Reactions (ii) and (iii) show that there is some component, mainly 
nuclear, which can react with $p$-nitrobenzoyl chloride (without water 
treatment) but which fails to react with benzoyl chloride. The water 
effect shows that this is a shielded component (in the frozen-dried tissue), 
and that when revealed, benzoyl chloride itself is capable of reaction with 
it. This is not necessarily surprising, since the more activated $p$-nitro-
benzoyl reagent might compete more effectively with a bonded group at 
the acceptor site. Reaction (i) shows that the reactive component is not, 
however, the same as that responsible for the BDC reaction. This is 
confirmed by the result of a further experiment:

(iv) Benzoylation followed by coupling with diazotized sulpha-
nilic acid (cf. Section 5 below) and then by $p$-nitrobenzoyl chloride 
used chromatogenically, again produces nuclear staining similar to the 
BDC reaction. Hence, the site of coupling after benzylation is not 
the site of $p$-nitrobenzoyl reaction. It should be noted here that the 
reduction stage in the colour development removes the azobenzene-
sulphonate groups (as shown by controls); their colour does not interfer-e, 
therefore, but the sites to which they were attached remain 
vacant.

The identity of the component concerned here is still unknown. It 
may be surmised that after benzylation shielded groups exist in the 
nucleoprotein other than those within the limited range of the diazonium 
reagent, and that one or more of these groups is revealed by this more 
reactive acylating agent. Nucleoprotein arginine groups do not appear
to be involved, since they do not show the water effect with benzoyl chloride, and since it has been observed (v) that \( p \)-nitrobenzoyl chloride, with or without prior water treatment, does not (at least qualitatively) block the nuclear (benzoyl chloride-resistant) Sakaguchi reaction for arginine.

c. Other Reagents

Trials with a few other acylating (and related) agents on several tissues have been made, only to the extent of showing further variations in reagent reactivity, with semi-qualitative assessment by eye only. \( p \)-Iodo-benzoyl chloride (3·2%) and 3:4:5-triiodobenzoyl chloride (6%) (applied in benzene, for solubility reasons; these reagents were synthesized by Dr. M. M. Coombs for electron microscope staining studies) give little or no blocking (up to 20 hr) of the \( p \)-nitrobenzoyl chloride colour reaction or of the tetrazo coupling reaction, the triiodo compound being the least reactive of the two. It seems likely that iodinated reagents of this particular type have severe limitations due to insolubility in suitable solvents, and to low reactivity. Phenyl isocyanate (10%, in pyridine, 2 hr) gives considerable blocking of both colour reactions but leaving a strong nuclear (and weak cytoplasmic) reaction. 3:4:5-triiodophenylisocyanate (6%, in benzene, up to 20 hr) gives no apparent blocking. \( m \)-Nitrobenzene sulphonyl chloride (5%, in pyridine-acetonitrile, 3 hr) used chromogenically gives a rather weak reaction at all sites, but red cells react strongly; the reaction is completely blocked by benzoyl chloride. Similarly, \( p \)-toluene sulphonyl chloride (7·5%, 3 hr) produces rather feeble blocking of the two colour reactions. Isocyanates may prove useful, but a relatively slow rate of reaction is apparently shown by the sulphonyl chlorides. Their stronger reaction in red cells, where histidine concentration (from haemoglobin) is high, may indicate preferential reaction at histidine groups.

5. Use of Alternative Diazonium Compounds

The use of tetrazotized dianisidine in the standard BDC method is dictated, as noted earlier, by the need for an intense colour in the reaction product. It is of interest to enquire whether the characteristic behaviour observed with this method is dependent on the particular diazo reagent employed.

Reaction with each of the diazotized amines, sulphanilic acid, \( p \)-nitroaniline and \( \alpha \)-naphthylamine (0·02 M, pH 9, 5°, 20 min) has been found to produce a weak yellow colour, which appears to show the same distributions, with and without prior benzoylation, as the corresponding colour reactions obtained with dianisidine. Moreover, when each of the
three mono-diazo reagents is applied, either with or without prior benzoylation, and is followed by the dianisidine coupling procedure, the intense red-purple colour reaction from the latter appears to be entirely blocked, only the initial pale yellow colour being observed. Hence, these three different diazonium compounds appear to react at the same sites, in the benzoylated and unbenzoylated cases respectively, as tetrazotized dianisidine (though measurements to test for an exact equivalence have not been made, and would be difficult to obtain with the required sensitivity).

III. INSTRUMENTATION AND PROBLEMS OF MEASUREMENT

A. MICRO-SPECTROPHOTOMETRY

It would be out of place here to discuss in detail the requirements and methods available for cytochemical micro-spectrophotometry with visible light. Recent pertinent reviews include those of Swift and Rasch (1956), Leuchtenberger (1958) and Walker and Richards (1959).

The distribution within a cell nucleus of the stain produced by the BDC reaction is often not homogeneous, and in general resembles that of the Feulgen stain. There will accordingly be the risk of distributional error (discussed, for example, in the reviews just cited).

One solution to this problem is that of Deeley (1955), involving the use of automatic scanning of the field by a small aperture. The Deeley apparatus, which employs mechanical scanning and electronic integration of the signals, has been used in all the measurements discussed here of the BDC stain. At the same time, the crushing condenser of Davies et al., (1954) has been employed in these measurements. With this latter device, the nucleus in question is flattened to any required degree by pressure applied through a sheet of cellophane. The combined effect of these measures is (a) to reduce local high absorbances to values which permit accurate measurement, (b) to minimize distributional error, and (c) to minimize out-of-focus errors.

Other solutions to the optical problems could no doubt be applied for measurement of this stain. In particular, the two-wavelength method (Ornstein, 1952; Patau, 1952) would be expected to be suitable. The use of a monochromator would in some respects confer an additional advantage since measurements could then be made at the absorption maximum, and different coupling components (with different $\lambda_{\text{max}}$) could be compared.

With the Deeley apparatus, filters isolating a fairly narrow spectral band are used. Using tetrazotized dianisidine and H acid as the coupling components, filters 77A and 58 (Wratten) are suitable. Other requirements (light source, etc.) are as for Feulgen micro-spectrophotometry.
Methods used in Feulgen micro-spectrophotometry are not necessarily sufficient, without modification, for all cases in protein cytochemistry.

In the present state of techniques, micro-spectrophotometry is most usefully applied, in the case of a nuclear component, to the determination of the total amount of that component per nucleus, i.e. the entire nucleus is taken, as a readily identifiable and physiologically distinct entity. Various methods of estimating this quantity have been employed. Measurement of the stain contained in a plug within the nucleus has often been used, but this requires assumptions about the homogeneity of the nuclear stain and about the shape of the nuclei, both limiting the scope of application. These assumptions are not required in the measurement of entire nuclei by either the scanning technique or the two-wavelength technique. However, in all the methods two additional problems are met in the measurement of the total stain per nucleus:

1. Cut Nuclei in Tissue Sections

To minimize errors from this source, it is necessary to cut sections thick enough, and to rely on the recognition of any cut nuclei present. This creates difficulties due to the overlap of nuclei (see below) in a number of tissues.

2. Stain in Overlying, Underlying or Adjacent Regions

This becomes a major problem in protein cytochemistry, in the cases where the stained component is present in the cytoplasm as well as the nucleus. Even for a stain present in the nucleus only, difficulty still arises from this source from near-by, out-of-focus nuclei in thick sections. In some cases, e.g. spleen or thymus, overlap of nuclei is always serious.

To measure the total nuclear stain by either the scanning or the two-wavelength methods, it is necessary to have an unstained region surrounding the measured nucleus, in order that the image of the nucleus may be totally enclosed within the diaphragm without contributions from extraneous absorption. Further, the background reading, giving the incident light $I_0$ over the same field, should be made on a clear area as close as possible to the original area to avoid variations in $I_0$ due to variation in the specimen, the mounting conditions, etc. These requirements at present greatly restrict the application of these two accurate methods of measurement when the protein component is not confined to the nucleus. Failing an advance in photometric technique, these cases can be tackled by some method of isolation of the nuclei, providing significant losses are not thereby incurred.
In the case of an exclusively nuclear component, as with the BDC stain in most tissues, the last-named requirement can often be satisfied in tissue sections, if overlapping and cut nuclei can be carefully excluded. But the crushing condenser method, as used with the Deeley apparatus, cannot be employed with most sections of the required thickness, since overlap becomes prohibitive on crushing.

For the reasons mentioned above, tissue smears containing separated whole cells or disrupted cells have been employed here (see p. 226), thus ensuring that entire and spatially separated nuclei are measured. Smears have been found preferable in certain cases previously for similar reasons, in Feulgen or arginine micro-spectrophotometry (e.g. Ris and Mirsky, 1949; Swift, 1950; McLeish et al., 1957; Richards et al., 1956). An additional advantage of tissue smears is that the nuclei are normally rather flattened and the cytoplasm can be dispersed thinly, thus reducing scattering errors. Smears carry the disadvantage, of course, that the organization of the tissue is lost. The method is valuable at present for giving an adequate survey of classes of nuclei from a tissue, but in later, more sophisticated studies, some means of utilizing the information contained in the structure of the intact tissue will doubtless be required.

C. MOUNTING REQUIREMENTS

Three main factors have determined the choice of mounting medium:

(i) the normal optical requirement for a mountant, that the refractive index is sufficiently close to that of the tissue to reduce light scatter to a negligible level;

(ii) extraction or diffusion of the stain must not occur in this medium;

(iii) for use in the crushing system, the medium must facilitate the flattening of the nuclei when they are sheared.

For measurements of the Feulgen stain with the Deeley apparatus, mounting in glycerol is normally employed. With the BDC stain, glycerol has been found excellent with respect to requirement (iii), adequate for (i), but gives appreciable, though slow, extraction losses.

Several other possible media have been tested. Immersion oil (Shil-"l"aber's) is excellent for (i) and (ii), but flattening is inadequate in this medium, and similarly in liquid paraffin (see Fig. 5). It is not entirely clear what properties the medium must possess for a crushed nucleus to show the full flattening effect. Normal lubricating properties alone are not sufficient as is shown by the results with the two last-named media and, further, with a fluorinated hydrocarbon lubricant (Perfluorolube
oil), which gives partial, but still inadequate, flattening. Light scatter is also higher with this medium, although if this were the sole defect it might be countered by an additive of suitable refractive index. No medium has yet been found (though the search has not been exhaustive) which gives the same desirable effect in the crushing method as glycerol, where nuclei can readily be flattened one by one to any required degree down to a thin sheet. Some molecular interaction between tissue components and glycerol is presumably involved, perhaps related to the swelling of proteins by glycerol (Caspersson, 1950). The elastic properties of the cell must be changed in such a manner as to allow plastic flow to occur when stressed. However, detectable losses in stain occur on standing in glycerol (Fig. 5), again possibly related to the protein swelling effect. Fortunately, these losses are slow enough to allow measurement to be made in this medium.

The best procedure is to store the specimen in immersion oil from the time of staining until measurement, and then to wash and transfer to

![Fig. 5. Distribution of BDC stain in nuclei of mouse liver cells, as measured in various mountants. The same specimen was measured in turn in paraffin (P), glycerol (G.1) and again after standing 4 days in glycerol (G.2). Only nuclei apparently tetraploid (by approximate relative size) were measured. Another specimen from the same animal was measured in perfluorolube oil (PFL); approximate size ranges are shown. Crushing was observed to be fully efficient only in the glycerol case. (Cf. Fig. 14B for full distribution in mouse liver.) For comparison with G.2, a specimen of mouse kidney, mean 59.9 ± 1.2, showed a decrease to 44.3 ± 1.2, after 7 days in glycerol at 0°.](image-url)
glycerol. Readings are reproducible up to at least 10 hr in glycerol, but after about 2 days losses begin to occur slowly. A correction cannot be applied for these later losses, since they are detectable in some cells but not others in the same specimen, and they vary with the tissue and with the specimen treatment. It has been confirmed on all tissues used that it is safe to measure in glycerol throughout the first day of mounting, since the same cells measured initially and finally give unchanged values. Further, in cases where the nuclei do not contain regions of very high extinction and are reasonably flat initially, mounting in paraffin or immersion oil can be satisfactory since crushing is then not necessary; in these cases, the same values are obtained as subsequently in glycerol, confirming that no initial extraction occurs in that medium. It is not safe, however, after measuring a specimen in glycerol, to wash it (in water, alcohol and xylene) and return it to immersion oil for storage, in order to continue measurements later: it has been found that some losses are detectable then even after such storage at 0°, presumably arising from bound glycerol.

IV. PROCEDURES, AND THE EFFECTS OF VARIABLES THEREIN

A. STANDARD PROCEDURES

1. Freeze Drying

Freeze-drying is the most desirable method for the preparation of the specimens, both on the theoretical grounds mentioned later and on the empirical ground that a high and reproducible level of reaction is thus attained.

For tissue sections, standard methods of freeze-drying (cf., e.g. Bell, 1956) small pieces of tissue are satisfactory. The problem of freeze-drying thin smears will receive attention here, since (i) some specimens, e.g. ascites tumour, must be examined thus, and (ii) more importantly, normal tissues can often with advantage be examined in smear form for exact micro-spectrophotometry. A small fragment of the tissue (e.g. liver, kidney, etc.) is tapped with a perfectly flat-ended rod in a little isotonic saline for disruption, and smeared on a cover-slip, which is immediately quenched in a stirred bath of iso-pentane : propane (1:2) at liquid nitrogen temperature. The pressure exerted during the tapping and the smearing determines whether nuclei are liberated, and to what extent, from the separated cells. The saline can be replaced by other media, e.g. Tyrode solution, sucrose solutions, etc., depending on the degree of retention required. No medium at all need be used, although free tissue fluids are always present; a smearing-squashing technique is then still
satisfactory for most tissues, but some thick regions may be present and must be ignored. In the BDC reaction, the intensity has been found in practice to be the same whether the nuclei are in intact cells, or liberated in saline or without external medium. A similar smearing technique has been used by B. M. Richards in Feulgen measurements of the DNA content per nucleus (cf. Richards et al., 1956).

The quenched smears are washed by dipping in liquid nitrogen and are rapidly transferred to the drying chamber (see Appendix 3). Drying is carried out at —50°, although any temperature below about —40° is permissible, the rate of drying decreasing with decrease in temperature. It is essential to maintain the smear surface at a fairly uniform temperature. Unevenly dried or poorly preserved specimens give a decreased or variable reaction. When dry, the smears are raised to room temperature in vacuo, and rapidly removed to a desiccator for storage.

The reaction cannot be obtained on smears or sections prepared by the method of freezing-substitution (Simpson, 1941) using absolute methanol or ethanol.

2. Specimen Pre-treatment

Smears for micro-spectrophotometry are simply fixed in absolute ethanol immediately prior to reaction. Frozen-dried tissue blocks are embedded in wax in vacuo and sectioned. Mounting on slides must be performed either by slight warming alone, or by flattening on acetonitrile or on 95% alcohol (e.g. at 47°, 30 sec). The water present in the latter medium does not appear to affect the wax-infiltrated tissue in these conditions. The wax is removed by xylene when required.

Fixation methods other than freeze-drying have not been studied in detail on account of their uncertain effects on the nucleoprotein. Tissue fixed in bulk in absolute alcohol, or in Lewitsky's fluid, gives the reaction, but to what quantitative extent is undetermined. After some fixatives, e.g. Carnoy's fluid, the reaction is weak or variable.

3. Reaction Method

(i) Fixation in absolute alcohol, two baths . . . 20 min
(ii) Wash in dry acetonitrile, two baths . . . 6 min
(iii) Benzylation: Dry acetonitrile (50 ml), benzoyl chloride (4·2 ml) and dry pyridine (2·2 ml). Used at room temperature, in a desiccator (CaCl₂) . . . . . 3 hr
(iv) Alcohol wash, two baths . . . . . 6 min
(v) Take down to water through 90, 70, 50 and 25% alcohols.
(vi) **Coupling** (all operations in this stage are performed in an ice bath; specimens must be at 2°-4° throughout).

Wash in ice cold water .................................. 4 min

*Tetrazotized dianisidine*: a solution (0.04%) of the stabilized salt (Appendix 2) in sodium veronal (2%) buffer, pH 9.0. This solution is made immediately before use and filtered quickly in the cold through a coarse paper. It is a clear yellow solution, which darkens on standing. Normal exposure is 18 min.

*Washes*: Veronal buffer (0.2%, pH 9) ................ 1 min
Water, two baths ........................................ 1 min each
0.05 N HCl .................................................. 1 min
Water, two baths ........................................ 1/2 min and 1 min

*Second coupling*: H acid (0.1% solution of the Na₃salt) in carbonate-bicarbonate buffer, pH 9 .................................. 10 min

*Or* β-naphthol (0.1%) in Na₂CO₃ solution (0.5%) .... 10 min

The specimens are then allowed to warm to room temperature, in the second coupling bath, over a period of 20 min.

(vii) Wash in Na₂CO₃ solution (0.5%), two baths .......... 6 min
Wash in water, two baths ................................ 6 min

(viii) Dehydrate through 25, 50, 70 and 90% alcohols.

Absolute alcohol, two baths ................................ 6 min
Alcohol-xylene (1:1) .................................... 1 min
Xylene, two baths ....................................... 6 min

(ix) Mount in balsam, or for measurement mount and store in Shillaber's immersion oil under cellophane.

Some of these periods are flexible (see B, 3 below). Precautions must be taken to ensure that no moisture comes into contact with the specimen from the stage of freeze-drying to the end of benzoylation.

4. **Micro-spectrophotometric Method**

Measurements on this reaction have so far been carried out using the scanning, integrating micro-spectrophotometer of Deeley (1955). The procedure used, described below, is similar to that of Deeley *et al.*, (1954) and of Richards *et al.*, (1956) for measurements of Feulgen stain. Other micro-spectrophotometric methods, of proven reliability elsewhere, can no doubt be applied here.

The specimen (here, in all cases, a smear on a cover-slip) is taken from the temporary mounting in immersion oil, washed in xylene and alcohol and in 90, 70, 50 and 25% alcohols and water, and drained. It is mounted
in glycerol under cellophane, and secured to a brass holder by wax. Objective and condenser lenses are immersed in Shillaber's oil. Nuclei are crushed by the crushing condenser, then totally enclosed by the diaphragm, and measured. The reading, at the same diaphragm setting, for an adjacent blank area is subtracted, to give the integrated total absorption (in arbitrary units) for that nucleus. The mean of three readings is taken in every case.

Nuclei are measured in a number of different areas selected at random on the same specimen. Where significant variability among similar cells is found between different regions of one specimen, it is discarded: this can occur if drying was not satisfactory and is usually correlated with inferior cytological preservation.

B. EFFECT OF VARIABLES IN THE CYTOCHEMICAL PROCEDURE

In establishing the conditions giving the most satisfactory and reproducible results with the maximum production of colour, frozen-dried rat liver and kidney, and chicken or frog blood smears have been used, in both qualitative and quantitative observations.

1. Coupling

Reaction using o-dianisidine gives, as expected, a rather stronger colour than using benzidine. pH around 9 seems optimum; adsorption of decomposition products becomes appreciable at higher pH, necessitating further washing with the risk of some loss of the free diazo group.

![Graph](image-url)

**Fig. 6.** Variation of BDC staining with length of exposure to standard tetrazo solution (concentration of free tetrazonium dichloride, 0.017%). Frog erythrocytes; each point represents the mean stain (± S.E.), in arbitrary units, for about 30 nuclei.
Concentrations of (free) tetrazotized dianisidine much above 0·02% also incur this danger. The wash with dilute HCl is intended to destroy the acid-labile triazenes (cf. Section II, A). The concentration used, 0·05 N, is that which was shown, in work on tissue in bulk, to be the lowest that would give maximum splitting at those sites.

With other conditions standardized, the extent of reaction has been measured, in the nuclei of frog red cells, as a function of the exposure to the tetrazonium reagent (Fig. 6). The stain increases with length of reaction time to reach a plateau of maximum stain. At 60 min, however, there are signs that decomposition products are accumulating. Fourteen to 30 min is optimal.

Of the naphthols tried (Table I), H acid gives a reaction product with the most suitable light absorption properties for micro-spectrophotometry of the present type. In measurements on frog red cells, it has been found that the intensity is unchanged for exposures to H acid solution, from 4 min to 12 min at 2°, followed by warming up to room temperature, the total period in H acid being constant at 30 min. Coupling in H acid immediately at room temperature, however, gives a decrease (15%) in mean intensity.

2. Benzoylation

Qualitatively, the same pattern of dependence on length of benzoylation has been observed on a number of different tissues, namely the cytoplasmic stain (apart from the special cases described in Section VI, B) decreases rapidly over the first 30 min and appears very slight or negligible at 1½ hr and nil thereafter. The nuclear stain persists after at least 20 hr benzoylation. The concentration of benzoyl chloride can be reduced to as low as 1·5%: 18 hr treatment then gives the same result as 10% benzoyl chloride for 3 hr.

Quantitatively, the conclusions with regard to time dependence have been confirmed in the case of frog red cells (Fig. 7). It is seen that from 2 to 20 hr the nuclear stain remains constant. Cytoplasmic stain is zero at 2 hr. Hence the difference in reactivity involved is not merely kinetic, but is so great as qualitatively to distinguish this nuclear component. Measurements after less than 2 hr benzoylation are complicated by the cytoplasmic stain remaining, i.e. the nuclear stain cannot be measured in whole cells by the present method without the inclusion of stain (where present) in some adjacent or overlying cytoplasm. It might be of value to measure nuclei isolated by the non-aqueous method (Allfrey et al., 1952) to examine the initial total nuclear stain and its change with benzoylation. Routinely, 3 hr benzoylation is satisfactory. Exposures as long as 20 hr give detectable swelling of some structures.
Benzoyl chloride (10%) in dry pyridine alone gives some blocking, but the medium develops a strong red-purple colour which tends to stain parts of some tissues. This colour is similarly obtained with rigorously purified pyridine, and is due to an undesirable secondary reaction. This is reduced to a low level in the acetonitrile medium. Benzene and similar vehicles have also been tried, but a precipitate of the quaternary benzoyl salt of pyridine is present in these. The omission of a tertiary base gives, as expected, slow and inefficient benzoylation. Trimethylamine can replace pyridine, but secondary reactions, forming a colour in the medium, are more pronounced with this base.

Controls, subjected to the acetonitrile-pyridine solution with benzoyl chloride omitted, have been applied in all tissues examined, and give a subsequent coupling reaction identical with that obtained in an untreated parallel specimen.

3. Other Variables

Micro-spectrophotometry of frog red cell and mouse kidney smears has shown that no detectable change in the amount of the BDC stain occurs with (a) extension of the initial alcohol fixation up to at least 24 hr; (b) length of time in alcohol after benzoylation up to at least 20 hr; (c) length of time in water before coupling, up to at least 2 hr; (d) length of time in alcohol after coupling, up to at least 3 hr; (e) length of storage in Shillaber's oil, from nil to at least 1 month (at 0°C).
C. Procedures for the Establishment of the Chemical Basis of the Cytochemical Reaction

The methods described in this section have had as their object the reaction of nuclei in bulk, followed by fractionation, separation of a fraction containing the introduced dye groups, and analysis to identify the sites of reaction. Such methods should be applicable, with appropriate modifications, to the analysis of other reactions of this type (cf., for example, Maddy, 1961). Chicken erythrocyte nuclei were chosen as material in the present case, on account of their ease of preparation and their homogeneity. Other material could often, no doubt, be employed with advantage.

1. Preparation and Reaction of the Material

In a typical preparation, chicken erythrocytes were obtained from whole blood (about 200 ml) by centrifugation and washing in isotonic (0.93%) saline. Freezing and thawing has been found to be the most satisfactory method of preparation of uncontaminated nuclei. Five consecutive thawings are required, and the temperature of the suspension must be kept below 5°. After four cold saline washes, to remove some attached stroma, the nuclei appeared in good condition, could be gelled, and smears fixed in alcohol showed normal Feulgen and BDC reactions and the water effect. The nuclei were suspended in cold saline (40 ml) and injected in a fine, rapid stream into cold, stirred ethanol (300 ml) avoiding formation of gross clumps. The fixed nuclei were washed with ethanol, and shaken in ethanol (360 ml) for 2 hr at room temperature. After two washes in acetonitrile, they were shaken for 4 hr in a flask containing dry acetonitrile (250 ml), benzoyl chloride (20.6 ml) and pyridine (14.1 ml).

Washes were given in alcohol (2), in 90%, 70%, 50% and 25% alcohols and in cold water (3). The nuclei were stirred for 20 min in a cold solution of tetrazotized dianisidine (0.06% of the stabilized salt) in 2% veronal buffer, pH 9 (360 ml) at 4°, and centrifuged. Washes (with centrifugation for 2 min only, at 1900 g) were given in veronal buffer, water, 0.05 N HCl and water (2), followed by stirring for 15 min in 0.15% R acid in 0.5% NaHCO₃. All operations here were at 4°. The nuclei were then allowed to warm to room temperature for 15 min, while stirred in R acid solution. The product was washed with water (3), 1% Na₂CO₃ (3), 0.01 N NaOH and water (2). A marked difference in intensity of benzoylated and parallel unbenzoylated, coupled nuclei is apparent after coupling.

The effects of variation in the washes at each stage have been examined to establish that the procedures are adequate. Thus, maximum attainable removal into the supernatant of triazenes and of excess
ACYLATION AND DIAZONIUM COUPLING

reagents has been demonstrated, in addition to the removal, by the prescribed washes, of adsorbed similar dyes added in test experiments.

2. Fractionation of Reacted Nuclei

The benzoylated, coupled nuclei were washed in ethanol-ether (3:1, 360 ml), removing about 4% of the total dye present. This material has the properties of a lipo-protein or a very highly benzoylated protein fraction. Repeated washes gave no further removal. Two similar extractions of 10 min each under reflux were given, without further colour loss, to remove lipid. A preparation dried in vacuo at this stage yields about 1·9 g from 180 ml original blood.

TABLE III

TYPICAL COURSE OF EXTRACTION OF NUCLEIC ACID FROM SAMPLES OF REACTED, LIPID-EXTRACTED NUCLEI

(A) Extraction in N HCl, 100° (0·47 g tissue)

<table>
<thead>
<tr>
<th>Total hydrolysis period (min):</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>20c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance (5 mm cell, 265 mµ)</td>
<td>36·2b</td>
<td>0·575</td>
<td>0·197</td>
<td>0·090</td>
</tr>
<tr>
<td>λ max (mµ)</td>
<td>265</td>
<td>228</td>
<td>228</td>
<td>228</td>
</tr>
</tbody>
</table>

(B) Extraction in 5% trichloroacetic acid, 90° (1·01 g tissue)

<table>
<thead>
<tr>
<th>Total hydrolysis period (min):</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>32</th>
<th>38</th>
<th>44</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance (5 mm cell, 265 mµ)</td>
<td>72·0b</td>
<td>1·89</td>
<td>0·852</td>
<td>0·692</td>
<td>0·386</td>
<td>0·239</td>
<td>0·195</td>
</tr>
<tr>
<td>λ max (mµ)</td>
<td>265</td>
<td>265</td>
<td>269</td>
<td>269</td>
<td>269</td>
<td>269</td>
<td>269</td>
</tr>
</tbody>
</table>

a 50 ml fresh, hot acid applied (under reflux) for each interval, followed by quenching. Washings (30 ml) are added; final volume 100 ml. The times are recorded cumulatively.

b Deduced from reading after 100-fold dilution, assuming Beer's Law applies.
c Subsequent visible absorption (Spekker) measurement on the nuclear dye material taken into alkaline solution, showed no significant loss (on an initial weight basis) compared to the product from non-acid-extracted nuclei.

Nucleic acids were extracted by N HCl (3 batches) under reflux, the sulphonated dye-linked protein remaining insoluble. The release of material with an absorption peak near 260 mµ was followed (Table III), to show that nucleic acid removal was virtually complete in 20 min. Trichloroacetic acid (5%) gives the same result but more slowly. No dye was lost in these removals.
Extraction with $0.7\ \text{N NaOH (80 ml)}$ under reflux, 12 min, gave an intensely red-orange solution, and an insoluble residue containing a very small percentage of the total dye. On titration, the solution changes to purple-blue at pH 10, and the dye-linked fraction is selectively precipitated around pH 6 to 5. Repeated re-precipitation freed it from some uncoloured proteins. The fraction obtained, containing, in covalent linkage, almost all the initial colour, is protein in nature, yielding on reduction, hydrolysis and chromatography a typical amino acid mixture, without purines, pyrimidines, sugars or any other obvious additional component.

3. Hydrolysis of the Azo-dye-linked Protein

A number of proteases, used singly or in combination, were ineffective in hydrolysing this material, presumably due to the benzoyl groups (Bergmann and Fruton, 1941) and dye groups present. Acid hydrolysis (6 N HCl) gave quite rapid destruction of the dye group, while reflux in 5 N NaOH gave slower, but still prohibitive, dye loss. Similar synthetic dyes alone show similar, though rather slower, destructions.

The solution finally found to the problem of cleavage of the peptide bonds with conservation of the azo links involved hydrolysis catalysed by a cationic detergent. Protein hydrolysis catalysed in dilute acid by anionic detergents has been shown by Steinhardt and Fugitt (1942): the use of a cationic detergent in alkali was investigated here because of the affinity of such a detergent for the sulphonic groups in the dye radical, the lower sensitivity of these dyes to alkali, and the desirability of conserving tryptophane. The best medium tried was $0.015\ \text{M cetyl trimethyl ammonium bromide (CTAB) in 0.3 N NaOH}$, under reflux, which gave very small dye loss and eventual complete hydrolysis of the protein.

4. Fractionation and Identification

The group(s) $X$ linked to the dye radical in the tissue must be released on hydrolysis to give the dye compound IV.

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{OCH}_3\text{OH} \\
\text{X} & \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \\
\text{SO}_3\text{H} & \\
\text{SO}_3\text{H} \\
\text{reduction} & \\
\text{X.NH}_2 & + \\
\text{NH}_2 & \quad \text{CH}_3\text{O} \\
\text{HO}_3\text{S} & \quad \text{OCH}_2 \\
\text{SO}_3\text{H} & \\
\text{NH}_2 & \quad \text{NH}_2
\end{align*}
\]
From the hydrolysate, the detergent-dye complex was salted out in strong alkali and dissolved in water. Ion-exchange chromatography (Zeo-Karb, 225/H⁺) with displacement by 0·2 N NH₄OH/50% ethanol, separated the dye compound. By paper chromatography of this and of the amino acids in the hydrolysate, the progress of hydrolysis was followed: 15–20 hr was sufficient for full release of IV. One main dye spot was obtained in several solvents, but no identification is possible by paper chromatography since a number of different bis-azo, sulphonated dyes of this type give very similar Rₚ values.

On reduction by hydrosulphite, and chromatography, o-dianisidine and 1-amino-R-acid were identified (cf. equation 2) and an unknown compound (NH₂-X, Table IV), positive to ninhydrin. Eluates of this unknown showed rising u.v. absorption below 240 mµ only, inconsistent with a tyrosine or tryptophane derivative.

Synthesis of 3-amino-tyrosine and 2-(or 4-)amino-histidine and comparison with NH₂-X (Table IV) supported a derivation from histidine. The two isomeric amino-histidines are unstable (cf. Fargher and Pyman, 1919; Diemair and Fox, 1938; Hunter and Nelson, 1941) and were prepared together in solution only, by reduction of pure mono-(p-azo-benzoic acid)-histidine. It appeared that one isomer only was obtained from the tissue derivative, but no orientation was possible.

Final confirmation was made by infra-red spectroscopic analysis, in collaboration with Professor W. C. Price, Mr. R. Bradbury, Dr. M. A. Ford and Dr. G. R. Wilkinson. The compound NH₂-X, obtained by elution after chromatography on thoroughly pre-washed paper, and deposited on an AgCl disc, gave an unusual infra-red spectrum (Barnard, 1957) showing interaction with groups in the cellulose, but similar spectra.

### Table IV

**Paper Chromatographic Behaviour of Dye Reduction Products**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rₚ (1)</th>
<th>Rₚ (2)</th>
<th>Rₚ (3)</th>
<th>Ninhydrin</th>
<th>Diazocoupling</th>
<th>Fluorescence</th>
<th>λ_max (mµ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂-X</td>
<td>0·18</td>
<td>0·10</td>
<td>0·34</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Amino-histidine</td>
<td>0·18</td>
<td>0·10</td>
<td>0·34</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Amino-tyrosine</td>
<td>0·16</td>
<td>0·30</td>
<td>+</td>
<td>Bright</td>
<td>+</td>
<td>270</td>
<td></td>
</tr>
</tbody>
</table>

Solvents: (1) Butanol: acetic acid: water (4:1:5)  
(2) Ethanol: ammonia: water (20:1:4)  
(3) Phenol: water (4:1)
were given by synthetic amino-histidine and by histidine itself (but not by tyrosine), after similar elution. This behaviour is given only after drying of the paper; it appears that histidine and its derivatives then form a spectroscopically distinct complex with a cellulose fraction, presumably related to the strong hydrogen-bonding properties of the system (Barnard and Stein, 1958). Elution in the original wet state prevented this phenomenon. However, the material then darkened during elution and deposition, and gave a different spectrum, containing bands characteristic of an amino acid, but suggesting self-polymerization had occurred (Barnard, 1957). Decomposition is minimized by chromatography in the dark in a reducing atmosphere, but still occurs in the solid state and when irradiated. The synthetic amino-histidine gave an identical spectrum, not obtained from any other source.

Thus, although two types of anomalous behaviour were found in the infra-red study due to peculiarities of histidine, both were shown in the synthetic and tissue derivatives, and these produced identical spectra in each case. The analyses left no doubt that X is histidine.

V. CRITIQUE OF THE METHOD

A. NATURE OF THE CYTOCHEMICAL REACTION

Criticisms have been made by one or two authors who report that they attempted to apply the original proposals of Danielli (1950, 1953) for benzoylation and for tetrazonium coupling. Gomori (1952) attributed the results to adsorption artefacts, while Burstone (1955) concludes that the tetrazo reaction is non-specific and difficult to interpret, and is completely abolished by benzoylation. These authors used chemical fixations and did not, apparently, benzoylate in truly anhydrous conditions. Burstone’s procedures differ in a number of significant respects from those used here; some of the qualitative results that he reports with tetrazo coupling, benzoylation and p-nitrobenzoylation are certainly contrary to those that I have found with the present procedures, and to some results of Maddy (1961).

The interpretation placed upon the observed staining in the present case rests upon (i) the results of analytical studies of reacted material, and (ii) a self-consistent set of cytochemical observations which support or extend this interpretation. However, the biochemical analysis, which is laborious, has been made in only one case so far, and even there no quantitative evidence exists to show that the amount of reacted histidine accounts for the total stain measured in the cytochemical specimen. Attempts at such a correlation would encounter the difficult problem of
deducing absolute amounts of material from micro-spectrophotometric measurements on cells. In these circumstances, it may be desirable, especially in view of the criticisms quoted, to summarize here the evidence that the observed reaction, examined in many cell types, is not affected by adsorption (whether of a dye-producing reagent or of a diffusible coupled tissue component) to any significant degree:

1. The behaviour is not changed when various naphthols (Table I) are used in coupling, although the dye products would then show varying adsorption behaviours.
2. Extension of various washing periods does not change the amounts of stain measured (Section IV, B).
3. Tests with synthetic dyes, derived from dianisidine coupled once or twice with either R acid or with β-naphthol, show, firstly, that these will stain unbenzoylated but not benzoylated sections. As expected, they behave as weak acid dyes, and the blocking of the tissue basic groups prevents their binding. Secondly, even when staining occurs thus, the final washing procedures normally used (including xylene washes when β-naphthol is used) remove virtually all of the adsorbed dye.
4. The results with other diazo reagents (Section II, D, 5) show that when all available sites for true coupling are blocked by any one of three different groups, excess unreacted tetrazo compound and any diffusible chromogenic products formed from it are removed by the washes given in the standard procedure.
5. The water effect, in which the occurrence of staining is determined by exposure to water at a remote stage, is not consistent with any known type of adsorption.

Pearse (1953) gives an interesting report. He found a distinct coupling reaction after benzoylation (in redistilled pyridine, 16 hr) in nuclei and many other sites, but that this was largely dependent on a “heat treatment” of the section at 80°. Such heating has not been found necessary in the present studies, nor does the degree of heat exposure in the embedding or mounting stages appear to affect the reaction. In fact, the reaction in the material used here is independent of any heating above room temperatures, as is shown by the results on air-dried and frozen-dried smears. The suggestion of Pearse (1953) that the reaction he observed was due to physical causes, involving a protein chain re-arrangement on heating, may be consistent with interpretations made here, the observed differences being due to differences in the pre-treatment of the material.

It must be stressed that the present interpretation of the reaction in terms of protective bonding has not been rigorously proved. It is the simplest and most consistent construction that can at present be placed
upon the evidence. A direct demonstration would come from the selective introduction of isotopically-labelled benzoyl chloride after water opening of the protected sites (at present under investigation). An attempt to use $p$-nitrobenzoyl chloride in such a demonstration (Section II, D, 4) failed, because of a reactivity difference revealed between this agent and benzoyl chloride.

The water effect itself might be thought to be paradoxical in view of the initial presence of the tissue fluids or of a saline wash, but it is, in fact, the exposure to water after the first dehydration of the tissue that appears to be operative; it must be presumed that it is in this initial dehydration that specific structural relationships are introduced in the nucleoprotein (see p. 250) which persist in anhydrous media only. The reaction can be obtained (qualitatively at least) after alcohol dehydration alone, and even in fresh frozen tissue sections fixed in alcohol. This, and the results on air-dried and frozen-dried smears, show that it cannot be dependent on lipid removal in wax embedding. The fact that the same measured value is found in frog red cells after simple air-drying and after exhaustive freeze-drying (in frog tissue smears) suggests that the structural alteration concerned reaches a stable limit after a mild desiccation.

The methods employed to show that the water pre-treatment does not remove the reactive histidine component are not competent to establish this in the case of the protecting component, if this latter is readily detached in water, although this may be thought to be rather unlikely.

B. The Analytical Procedures

The analysis has so far been made in one case only, that of alcohol-fixed chicken erythrocyte nuclei. It would be clearly desirable to make similar identifications in other tissues and, further, to use material frozen-dried and treated with alcohol as in the cytochemical case. The experience obtained with the chicken erythrocyte product should facilitate such further analyses. The use of such material would overcome another objection to the present analysis, namely the exposure of unfixed isolated nuclei to aqueous solvents, when considerable protein loss probably occurs. However, the reaction has been found to be unchanged quantitatively in mouse liver and frog erythrocyte nuclei exposed (5 min) to a saline wash; the reactive component would seem to be stably bound in the insoluble nucleoprotein complex. Refinement of the analytical methods to provide an accurate measure of the number of reactive histidine groups per nucleus, and of the ratio of this number to the total nucleoprotein histidine content, is also an important technical requirement for further investigations into the significance of the reaction.
The risks of distributional error, light-scattering and of cut or overlapping nuclei, and the measures employed to overcome them here, have already been discussed. There is some confidence in the instrumental method used since this appears to overcome distributional error in Feulgen measurements (Deeley et al., 1954; Richards et al., 1956) and gives consistent results here. It would, however, be of interest to make comparable measurements by the two-wavelength method, which has been shown to eliminate distributional error using a totally different method, for confirmation. A more severe requirement for the elimination of distributional error will come when stages of mitosis are examined.

A significant limitation of the present method is the enforced loss of topographical information in the tissue smears. This will probably necessitate some departure from or modification of the crushing method in future detailed studies.

No proof can be offered at present that Beer's Law is obeyed in the stained nuclei. The comparisons made in terms of "arbitrary units" between nuclei and between tissues are meaningless if considerable departures from this law occur. The spectral data obtained on similar dyes in solution (Section II, C) cannot establish Beer's Law up to the very high concentrations that may occur in small regions in nuclei (owing to the very small path lengths that would need to be investigated). However, in the scanning-crushing method employed the extinctions actually measured photo-electrically are all forced into a relatively narrow range, well below the higher values in the unflattened nucleus, and the effect of any departure from Beer's Law will therefore be minimized and is unlikely to be serious. It would, in principle, be desirable to obtain an independent check that significant departures do not persist, by comparing micro-spectrophotometric and biochemical measurements for a range of cell types.

D. Fixation Aspects

With regard to the cytochemical requirement for immobilization of the macro-molecules at their original sites, the advantages, in methods of the present type, of tissue preparation by freeze-drying are now widely recognized. No immobilization procedure can be ideal here. Even if significant diffusion is thus eliminated, there remains the second problem of the effects of the requisite treatment on tissue components; in particular, in protein cytochemistry of the present type, the effects on the reactivity of groups in various situations. While many chemical fixatives are particularly suspect because they may directly modify various protein groups by reaction, the effects of the protein structural changes implicit in any
fixation must also be borne in mind, and this is unfortunately true to some extent of the freeze-drying case.

The frozen-dried tissue specimen contains a macro-molecular solid matrix derived from the original, hydrated components. A subsequent brief fixation, e.g. in alcohol, is required to render this insoluble in water. This dual procedure is still superior to fixation in bulk, of course, for avoidance of diffusion and of gross modifications of proteins, but it must be recognized that the environment and reactivities of various side-chain groups (and their apparent accessibility) may be changed by the structural effects of the treatment. One piece of evidence for this is the irreversible loss in activity of certain enzymes on freeze-drying, a phenomenon which must be related to changes of this type. Freeze-drying is, therefore, an acceptable procedure in methods of the present type, but it will be necessary at some stage to enquire into the changes from the initial state that it has produced, in order both to obtain information on that state and to secure a fuller understanding of the reactivities actually observed.

VI. ASSESSMENT OF RESULTS TO DATE

A. THE PATTERN OF DISTRIBUTION IN TISSUES

As mentioned earlier, the characteristic result of the BDC reaction is nuclear staining (with an intra-nuclear distribution similar to that of the Feulgen stain in the same material) with the cytoplasm negative. This pattern has been observed in the cells of the following tissues (with exceptions, noted below, in the cytoplasm of certain cells):

**Rat.** Intestine (duodenum, ileum, colon), liver, kidney, stomach, pancreas, spleen, lung, trachea, salivary glands, skin, smooth muscle ( alimentary tract), skeletal and cardiac muscle, cerebellum, pituitary, adrenal, thyroid, thymus, testis, ovary, cartilage (hyaline and elastic), Walker carcinoma.

**Mouse.** Liver, kidney, Ehrlich ascites tumour.

**Frog.** Liver, kidney, intestine, stomach.

**Fowl.** Liver, kidney, lung.

In addition, red cells from rat, mouse and man are quite negative, while nucleated red cells (frog, fowl) show the normal positive nuclear reaction, as do leucocytes from all these species. Sperm (mouse, ram) show a weak reaction in the head, with a negative mid-piece and tail.

**Insects.** Testis (Locusta), salivary gland chromosomes (Drosophila).

**Protozoa.** Amoeba proteus.

**Plants.** Roots of Fritillaria, Hyacinth, Trillium, Vicia.
Fig. 8. Rat intestinal villus. Tetrazonium coupling *without* benzoylation. Magnification: $\times 870$. 
Fig. 9. Rat intestinal villus. Benzoylation and tetrazonium coupling. Only nuclei and goblet cells are positive. Magnification: × 870.

(Figs. 8 and 9: Serial sections, with photographic exposure and processing identical for each.)
Fig. 11. Rat pancreas. Benzoylation and tetrazonium coupling. Islet cells have negative cytoplasm, while basal zone in acini is positive. Magnification: × 120.

Fig. 12. Rat pancreas. Benzoylation and tetrazonium coupling. Details of cytoplasmic stain distribution in active acini are shown. Magnification: × 1000.
Fig. 13. *Vicia faba* root tip, showing dividing cells. Benzoylation and tetrazonium coupling. Magnification: × 900.
The generalization, therefore, appears to be widely based. No case of a frozen-dried tissue from any source failing to give the nuclear reaction has been encountered. Some typical results are illustrated in Figs. 8–13.

Cytoplasmic staining is found in a few specific sites, and would appear to fall into three classes:

(i) That related to high RNA concentration, as in the basal regions of pancreatic acinar cells (Figs. 11, 12). The region stained here coincides with the strongly basophilic zone, believed to be the site of considerable protein synthesis in zymogen production. Zymogen granules themselves, which usually lie towards the apex of the cell, and which are almost entirely protein, are negative after benzoylation. Some cells in the meristem of plant root tips similarly show cytoplasmic staining. Cytoplasmic reaction observed in some glandular cells in the pituitary (in particular in the pars anterior), and (weakly) in some active follicular cells of the ovary, in some proliferating regions of tumour tissue, and an occasional faint stain in some liver parenchymal cells is also thought to be of this type. Since cytoplasmic RNA must be present in a large number of cells not giving this cytoplasmic reaction, it is presumed that the exceptions here are due either to particular local aggregations of ribonucleoprotein, or to a specific bonding in the ribonucleoprotein at these sites only, perhaps related to intensive protein synthesis.

(ii) Cytoplasmic reaction occurs in the goblet and similar mucous-containing cells of the alimentary tract, in salivary gland cells and in granules in some cartilage cells. Extra-cellular staining occurs in some regions of cartilage matrix. There is evidence (Section II, D, 2) that at these sites the reaction arises from protein-carbohydrate bonding.

Both these classes of cytoplasmic reaction are abolished by the usual water pre-treatment, suggesting that labile bonds are involved. The pancreatic exocrine and plant meristem cells also show cytoplasmic shielded arginine groups (Barnard and Bell, 1960) again suggesting the bonding of protein to nucleic acid. In contrast, sites in class (ii) do not show the protected arginine reaction.

(iii) Extra-cellular fibres, apparently collagenous, are found to be positive at certain sites. These include a narrow band in the walls of distributing arteries and in the stomach and intestinal walls, but many collagen-type fibres are negative, as are basement membranes and typical elastic tissue. The reaction might conceivably be due to bonding of protein to the carbohydrate usually associated with collagens; but it is distinguished from the previous classes in that it is unaffected by water pre-treatment. The same is true of a reaction at these collagenous sites observed with p-nitrobenzoyl chloride after benzoylation. Perhaps a true reactivity difference is involved, or possibly this is one case where an
adsorption of the reagents occurs, specific to these sites, in view of the high adsorption affinity of collagenous structures. If so, washing to an extent greater than required for other sites may prevent this staining (the washing procedures not having been checked against the behaviour of these particular sites); but the reaction of these collagen fibres has not been studied in detail.

**B. Quantitative Results**

In frog (*Rana temporaria*) erythrocytes, the nuclei show a stain distribution with a single mode, at about 60 units, with small spread (S.E. 2–4% within one specimen) (Barnard, 1960a). In frog white cells of various types, in contrast, a very wide spread of values is found (from 60 to 360 units, in each of three males). The ploidy states of these latter cells are unknown.

In adult mouse liver, four classes are always observed (Fig. 14), of which the lowest appears to be derived from non-parenchymal cell nuclei (from connective tissues, bile ducts, blood vessels, etc.) and the other three, apparently from parenchymal cells, are presumed to correspond respectively to the diploid, tetraploid and octaploid classes present. In these parenchymal cells, there certainly appears, in practice, to be a rough correlation between the nuclear size class of a cell and its BDC
stain class, but accurate correlative volume measurements have not yet been made, nor has the DNA distribution in precisely the same nuclei yet been determined. The DNA distribution in liver parenchymal cells in similar mice is known, however, to be very similar to that found here (cf. Swift, 1950). Further, the liver of a young mouse (No. 5 in Table V)

**TABLE V**

**MEAN STAIN**a per NUCLEUS IN Mouse Liver CELLS

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Age (months)</th>
<th>Class IA</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>36.6 ± 1.9d</td>
<td>77.5 ± 2.4</td>
<td>149 ± 2.2</td>
<td>267 ± 7.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>40.8 ± 6.4</td>
<td>72.4 ± 4.7</td>
<td>138 ± 3.3</td>
<td>276 ± 12.5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>e</td>
<td>82.6 ± 2.8</td>
<td>146 ± 2.3</td>
<td>276 ± 5.5</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>f</td>
<td>41.7 ± 2.1</td>
<td>76.1 ± 1.5</td>
<td>138 ± 1.9</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>f</td>
<td>37.8 ± 2.1</td>
<td>58.4 ± 1.3</td>
<td>113 ± 7.4f</td>
</tr>
</tbody>
</table>

a BDC stain in standard arbitrary units (mean of 50–100 cells for each animal).

b C57 mice, males.
c Apparent non-parenchymal cells (see text).
d Mean ± Standard Error.
e Present, but not measured.
f Class III absent, and few Class II present; Class II value here drawn from only 3 cells.

showing little polyploidy cytologically, gave a BDC stain distribution very largely confined to the two lowest classes of Fig. 14.

The assignment here of parenchymal and non-parenchymal cell types respectively, is only approximate. It may be that some of the larger non-parenchymal nuclei are included in the second, "diploid parenchymal" class and vice versa, due to difficulties in cell recognition in the smears.

Mouse kidney cells show a single mode (Fig. 14), but there is a considerable individual variation there (Table VI) apparently not correlated with age. These animals were maintained under identical conditions, feeding ad libitum (stock diet 18, J. Rank Ltd.), but a closer control of the intervals after feeding, or of other metabolic variables, might be necessary to determine whether these individual variations can be ascribed to physiological variations in the nuclear protein.

Two features stand out from the quantitative results so far obtained. Firstly, the amount of stain is constant within a cell type, within limits that are narrow in sperm and nucleated erythrocytes, and fairly wide in kidney. Perhaps this denotes a connection with cellular activity.
Secondly, the stain is roughly proportional to the DNA content within a cell type range (polyploid hepatic cells), but shows large deviations from this when different cell types are compared (Barnard, 1960b).

![Graph showing distribution of BDC stain in sample of mouse sperm heads.](image)

**Fig. 15.** Distribution of BDC stain in sample of mouse sperm heads (from Barnard, 1960b). Sperm from three C57 mice give an overall mean of 11.9 units.

**TABLE VI**

**MEAN STAIN$^a$ PER NUCLEUS IN MOUSE KIDNEY$^b$ TUBULE CELLS**

<table>
<thead>
<tr>
<th>Animal$^c$ No.</th>
<th>Age (months)</th>
<th>Mean$^d$</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>58.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63.6</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>51.8</td>
<td>1.6</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>83.0</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>56.7</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55.0</td>
<td>3.7</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>60.3</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>49.6</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.0</td>
<td>1.0</td>
</tr>
<tr>
<td>11 (S)</td>
<td>$2^{1/2}$</td>
<td>59.5</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.9</td>
<td>1.9</td>
</tr>
<tr>
<td>12 (S)</td>
<td>$2^{1/2}$</td>
<td>49.3</td>
<td>3.7</td>
</tr>
<tr>
<td>13 (S)</td>
<td>2</td>
<td>66.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^a$ BDC stain in standard arbitrary units.

$^b$ No distinction has yet been made between cortex and medulla regions.

$^c$ C57 males or Swiss T.O. males (S).

$^d$ Mean of 20–50 cells per specimen. Duplicate values are for repeat specimens from same animal, reacted independently.
As discussed earlier, the bonded histidine component is associated with the deoxyribonucleoprotein of the nucleus. Further, the nuclear reaction is found to persist when the more soluble proteins have been removed in saline washes, and (in mouse liver and frog red cells) to be unchanged in amount then. In dividing cells it appears to be confined to the chromosomes (Fig. 13). However, quantitatively it does not always run parallel to the DNA content; and there is some evidence for a correlation with the total protein content of the nucleoprotein of the nucleus (Barnard, 1960b).

The relation of these histidine groups to the nucleoprotein structure is as yet uncertain. Comparison with recent X-ray diffraction results (Wilkins et al., 1959) suggests that the labile bonding observed is introduced into the nucleoprotein in the drying process (Barnard, 1960a). This is consistent with the weak or negative reaction given by frozen-substituted material, where it is presumed that alcohol has replaced water without the crumpling and consolidation of the nucleoprotein structure apparently produced by drying. This view is supported by a recent observation that subsequent drying (from cold alcohol) of frozen-substituted smears can introduce the capacity for the BDC reaction. Their previous incapacity cannot, therefore, be due to the loss of some component in the low temperature substitution process.

Since the stain values are reproducible and consistent, the bonding, if introduced by coalescence of nucleoprotein strands, must arise from specific alignments in these. On this view, the bonding is regarded as interprotein in nature, but the evidence does not at present justify the exclusion of the alternatives of a histidine-DNA bond, or the participation of some other, e.g. lipid, component.

Some suggestion that lipid might be involved in plants comes from examination of *Vicia* and *Trillium* roots after Carnoy, Lewitsky or freeze-drying fixations (Barnard, Chayen and LaCour, unpublished). The BDC reaction is obtained in the latter two cases only; this can be correlated with the retention of a nuclear lipid component (cf. LaCour, Chayen and Gahan, 1958). However, it is not known what concurrent structural changes occur in these various fixations, and further evidence would be required to establish the participation of lipid.

The situation of these histidine groups in the nucleoprotein structure in the *in vivo* state is an unresolved question of considerable interest. Their assignment to identifiable protein fractions has not yet been examined. In summary, at present the most favoured interpretation is that these groups form hydrogen-bonding sites on protein surfaces, in
specific alignments in the nucleoprotein gel. On desiccation, the structure becomes coalesced with definite bridges forming at these sites. Re-hydration reopens these, while attempted further dehydration by alcohol on the fixed material does not restore the previous situation. Structural information on nucleoprotein is at present too meagre to justify a more detailed model. Groups other than histidine may also be present at these sites, but would remain undetected here. The p-nitrobenzoyl chloride evidence cited above suggests that this is so. Maddy (1961) has similarly found that some nucleoprotein tyrosine groups are in a protective bonding, but this appears to be related to the presence of lipid, and its relation to the histidine sites is unknown.

Specific hydrogen-bonding sites of this kind may be of importance in vivo in nuclear activities in the alignment of other molecules along the nucleoprotein strands, or in the condensation of chromosomes prior to division. A possible suggestion of some relation to the latter phenomenon is shown by an observation on frozen-substituted tumour cells (mouse Erlich ascites and rat Walker carcinoma). These show in resting nuclei the weak or negative BDC reaction characteristic of frozen-substituted material, but cells in mitosis show a normal strong chromosomal stain. It is not yet established whether all the dividing cells in all stages of mitosis possess this characteristic, but it would appear that the condensed chromosomes themselves possess bridged sites similar to those introduced in the interphase nucleoprotein gel by the removal of water.

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APPENDIX 1: REAGENTS


These are stored in dry, glass-stoppered vessels. The benzoylation mixture is prepared immediately before use. All glassware used must be carefully dried.

Benzoyl chloride. AR grade.

Alcohol. AR grade, stored in well-stoppered bottle. Discard after use daily.

Tetrazotized dianisidine. This can be prepared in situ, but equally satisfactory results are obtained with greater convenience with the solid, stabilized salt (Appendix 2), freshly dissolved before use.

H Acid. Commercial specimens vary, and if impure should be re-crystallized from hot water. Prepare fresh solution daily, and do not expose to sunlight.

Glycerol. AR grade.

APPENDIX 2: PREPARATION AND STANDARDIZATION OF STABILIZED TETRAZOTATE

Commercial stable tetrazotates might be found satisfactory, but each must be tested before being accepted for accurate work. Some samples contain dark decomposition products, or metals such as aluminium which may be undesirable. The pure zinc chloride complex, prepared as described below, is recommended. The zinc is removed by filtration after dissolving the salt, when required for use, in the pH 9 buffer.

The preparation is modified from that of Saunders (1936).

o-Dianisidine dihydrochloride (12.8 g) is dissolved in hot water (100 ml), and filtered while hot, if necessary. The clear solution is cooled with addition of conc. HCl (16 ml), and left 5 min in the ice-bath, when part of the base hydrochloride separates. With the temperature at 6°C, a solution of sodium nitrite (7 g) in cold water (about 30 ml) is added rapidly with stirring, giving a dark solution of the diazonium chloride. (Unless this procedure is followed exactly, part of the base forms the triazene, the presence of which is highly undesirable in the cytochemical reagent). The temperature rises to 9°. The mixture is left standing in the ice-bath in the dark, with occasional stirring, until a clear light yellow solution is obtained (this may take ½–1 hr). The solution gives a strongly positive test with starch-iodide paper. A solution of zinc chloride (7.5 g)
in a little water is added to the cooled solution, followed by sodium chloride (25 g) in portions, when the double salt is salted out (adding a little more ZnCl₂ and NaCl if necessary). The precipitate is collected, washed with a minimum of ice-cold water and partly dried by suction. It is mixed with anhydrous sodium sulphate (2 g) and dried in a vacuum desiccator, giving a yellow powder. Samples dissolved in water have all the properties of a freshly prepared solution of tetrazotized dianisidine. The salt keeps for several years when stored in the refrigerator.

FIG. 16. Variation of absorbance ($E$) of bis-azo-H acid derivative with amount of tetrazotized dianisidine dichloride (reacted in 25 ml solution, diluted 10-fold and read at 580 mμ, 1 cm cell). For the determination of this compound in the stabilized salt samples (data by Mr. J. Marbrook).

_Standardization._ For quantitative work, the true concentration of the tetrazonium compound in the solution used must be known. Standardization is normally only required once on a given preparation, and is carried out by dissolving a known amount of the salt in water in the ice-bath and adding excess (> 2 equivalents) H acid and carbonate-bicarbonate buffer (pH 10). After 20 min in the ice-bath, the solution is taken to room temperature and the absorbance is measured (after appropriate dilution) at 580 mμ. The concentration is then read from a standard curve (Fig. 16). The latter is obtained by the tetrazotization of various known amounts of pure o-dianisidine by the method described above (without the addition of the ZnCl₂ or NaCl), adjustment to neutrality in the ice-bath, and a similar addition of excess H acid and buffer, followed by the absorbance measurement. A linear relation over an ample range is observed. The amount of stabilized salt used in the cytochemical reaction should normally be chosen so as to give a final concentration of tetrazotized dianisidine dichloride in the region of 0·02%. The sample referred to in the text contains 44% free tetrazonium dichloride.
APPENDIX 3: AN APPARATUS FOR THE FREEZE-DRYING OF TISSUE SMEARS

This apparatus was constructed in collaboration with Mr. J. Marbrook, and with the co-operation of the Drying and Distillation Division of Edwards High Vacuum Ltd. Our aim has been to obtain a simple modification of the existing Edwards tissue dryer, which would permit convenient, controlled freeze-drying of smears for quantitative cytochemistry and autoradiography. It also permits rapid drying of tissue blocks.

_Smears._ The quenched cover-slips are placed in the brass holder (Fig. 17) while still in the quenching bath at liquid nitrogen temperature, resting in the grooves and with their entire blank surface in contact with brass supports. The holder is rapidly transferred, via a liquid nitrogen bath, to the drying chamber (Fig. 18) at $-78^\circ$, and lowered into the brass cup on which is wound the heating coil $G$. The glass finger $F$ is rapidly replaced and the system is evacuated. When the pressure is virtually constant, the main trap is cooled with liquid nitrogen, and about 15 min later the cold finger is filled with nitrogen. The distance between the cold finger surface and the smears (about 15 mm) can be adjusted by perspex collars and neoprene discs at $B$. The smears are maintained throughout drying at the chosen temperature, say $-50^\circ$, by the thermostatted heater. The temperature is measured during drying by the thermocouple, whose junction is at a small blackened brass plate soldered to the rim of the heating coil cup. The thermocouple (and hence the thermostat) is initially calibrated in terms of the true specimen temperature, by comparison with readings obtained in a preliminary test in which the thermojunction is fixed in the position on the holder normally occupied.

![Fig. 17. Holder used for cover-slip specimens. The upright brass plate supports are set at an angle of about 100° to the solid base. The central hook is used in effecting transfers.](image)
by the specimen. Tests show this temperature does not vary appreciably over the surface of the smear. The thermocouple itself is pre-calibrated in the normal way over the required range.

Routinely, for use in the BDC quantitative method, drying is allowed to proceed at $2-3 \times 10^{-3}$ mm for about 20 hr. Probably considerably shorter periods will give adequate drying of smears in most cases. At

![Diagram of freeze-drying apparatus](image)

Fig. 18. Simple freeze-drying apparatus, e.g. for attachment to manifold of Edwards tissue dryer by standard vacuum unions. A, Pirani gauge, type M.6 (re-sited). C, isolation valve DSIA. E, vacuum union. F, cold finger, seated on vacuum-tight neoprene ring B. Thermocouple wires (not shown) are connected through leads passing through the standard Edwards drying-tube top D. G, heater coil wound on brass cup (3 cm external diam.). H, thermostat (modified version).

intervals the trapped ice can be removed from the cold finger tip as required by removing the nitrogen by suction and adding acetone (cf. Patten and Hopkins, 1958); most of the ice is rapidly sublimed into the main trap (in practice, part is held at first on the upper walls of the drying chamber at $-78^\circ$), after which the nitrogen is replaced. This process produces a pressure spike, which is reduced to a very low, constant level when no more moisture has been removed in the last interval, showing that drying is complete. During the overnight period, if used thus, a Dewar flask with a ground glass joint entry inserted at the base is attached to the top of the cold finger, the whole assembly being filled with
nitrogen and lagged. Nitrogen, and later undisturbed cold air, thus remain to maintain the cold finger surface temperature. (The main trap is temporarily removed for such overnight operation, the envelope being replaced by a dry one, using the isolation valves).

At the end of drying, after a final moisture transfer and with acetone in the cold finger, the thermostat is switched out of circuit and the heater at $G$ brings the tissue up to room temperature (the thermocouple leads being switched to reverse in order to follow this, using a second calibration obtained as before). Air is admitted through a moisture trap at $-78^\circ$, and the holder is swiftly transferred to a desiccator.

*Tissue blocks.* Wax is de-gassed and allowed to solidify *in vacuo* in a thin brass bucket which will fit into the heater cap $G$. A wide inverted, truncated cone is cut from the wax, exposing a circular area in the brass base. Quenched small pieces of tissue are placed on this in the quenching bath, and are held down during draining and washing in nitrogen. The bucket is rapidly transferred to the drying chamber; further procedure is then as before. The cold finger tip is again arranged to be at a suitable distance, say 15 mm, from the drying tissue blocks. After sufficient drying (1–2 days is routinely used, but the minimum is less, and varies with the tissue), the tissue is embedded *in vacuo* using the heater. It is essential at this stage to be able to watch the embedding process, both to know when the wax is fully melted and to observe any bubbling at the tissue, denoting incomplete drying. For this purpose, the CO$_2$-alcohol bath is partially lowered at the end of drying, any moisture that is held on the upper walls being then transferred to the main trap. When embedding is complete, dry air is admitted and the tissue in wax is removed to a desiccator.

REFERENCES


