IDENTIFICATION OF SOME CYTOCHEMICAL REACTION PRODUCTS BY HISTORADIOGRAPHY

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ABSTRACT

The reaction products of cytochemical methods for acid phosphatase, carbonic anhydrase succinate dehydrogenase, and thiolacetic acid esterase have been identified by historadiographic methods in sections of kidney. The absorption of x-rays by these products gives information on their localisation when they cannot be seen readily in the light microscope. The requirements for successful demonstration of such reaction products and the possibley value of the technique are discussed.

INTRODUCTION

The technique of studying the absorption of x-rays by small biological objects was introduced by Goby in 1913 (17). The extension of these studies to histological sections did not take place, however, until some time later when Dauvillier mounted the specimen in close contact with the photographic emulsion and used soft radiations to obtain an image (9). Lamarque developed the method (26–28) and working in conjunction with Turchini (34) studied the x-ray absorption of normal and pathological tissues (29). They introduced the term historadiography.

Castel, Lamarque, and Turchini (4) investigated the distribution of elements of high atomic weight incorporated into tissues during life and Barclay (1) studied the natural distribution of iron in cells. Mitchel (31) impregnated tissues with silver and demonstrated their increased x-ray contrast.

Engström proposed that histochemical analysis of endogenous elements in histological sections could be done using x-rays of long wavelengths (10). This idea was developed intensively and the theory and practice of measuring the total dr-mass of tissues and their content of calcium, phos phorus, and sulphur was evolved (30). The technique was used to study the mineralisation of bone and other tissues (see 11).

Engström and Jakus (13) suggested that the amount of protein in cells could be measured after staining with phosphotungstic acid but encountered difficulties with adsorption of x-ray absorbing elements on other substances in the cells.

Previous investigations have demonstrated that it was possible to identify the chromium deposited at the sites of localisation of adrenalin and noradrenalin in the adrenal medulla stained by the chromaffin reaction (18). It was also shown that historadiography could be used to study the products of cytochemical reactions in the case of alkaline phosphatase (19–21).

The method has now been applied to study the reaction products of several cytochemical methods for localising enzymes and it is the result of this study and its implications which will be presented here.

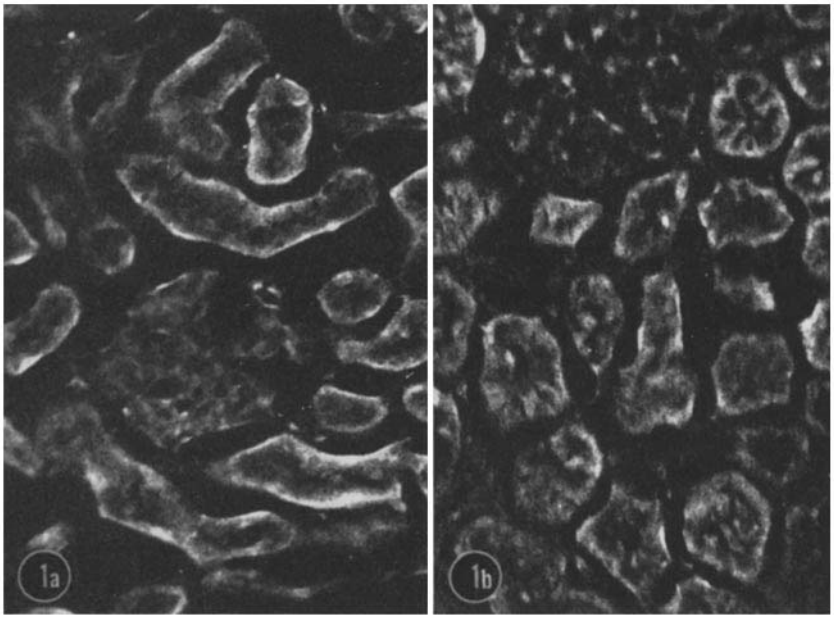


FIGURE 1

Historadiogram of (*a*) a fresh frozen section of rat kidney subsequently fixed in formalin; (*b*) a formalin-fixed frozen section of the same tissue. Both were exposed at 1.5 kv and 1.5 mA for 1.5 hours. × 250.

MATERIALS AND METHODS

Rat tissues were used throughout. The technique of preparation and examination of specimens has already been described (21). All specimens were exposed for 1.5 hours at 1.5 kv and 1.5 ma on Kodak maximum resolution plate.

The cytochemical reactions investigated were:

(1) The Gomori method for acid phosphatase as modified by Holt (24). Sections were removed and mounted on x-ray plate after incubation in the initial substrate and examined for deposition of the colourless primary reaction product, lead phosphate. Other sections were examined after conversion of lead phosphate to black lead sulfide by immersion in a week solution of ammonium sulfide.

(2) The Kurata method for carbonic anhydrase as modified by Hausler (22). Sections were examined after incubation in the initial substrate when the colourless primary reaction product, cobalt carbonate, had been formed. Other sections were examined after conversion of cobalt carbonate to black cobalt sulfide by immersion in a weak solution of ammonium sulfide.

(3) The MTT-cobalt method for succinate dehydrogenase of Pearse (33). Fresh frozen sections were mounted on celloidin-covered slides and incubated until a distinct colour reaction was obtained. The thin layer of celloidin together with the section was then stripped off and mounted on x-ray plate. An attempt was made to see if the x-ray absorption of this reaction product was attributable to the organic or metal component of the cobalt-formazan reaction product by comparing it with the non-chelated Nitro-BT reaction of Nachlas *et al.* (32).

(4) The thiolacetic acid esterase method of Crevier and Bélanger (8). Sections were examined after incubation in the substrate when the primary reaction product, lead sulfide, is deposited.

CONTROLS. Unincubated sections and sections incubated in the medium without substrate or in substrate without added calcium, cobalt or lead ions were examined by the x-ray method.

RESULTS

In two of the cytochemical reactions studied there is more than one reaction product. In these instances, the primary reaction product is colourless and cannot be seen in the light microscope. The secondary (final) reaction product may be coloured or opaque and will then be visible in the light microscope.

The historadiographic image of formalin-fixed and fresh frozen unstained control sections of rat

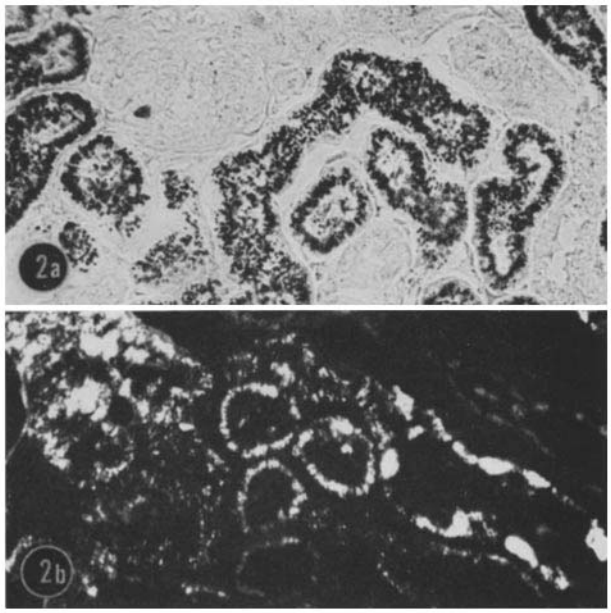


FIGURE 2

Pictures of the final reaction product, lead sulfide, in the method for acid phosphatase, obtained (*a*) in the light microscope, and (*b*), the x-ray system. Exposed for 1.5 hours at 1.5 kv and 1.5 mA. × 250.

kidney are shown in Fig. 1. A comparison of the conventional light microscope picture of the final black reaction product, lead sulfide, of the acid phosphatase method and its x-ray absorption picture is shown in Fig. 2. A more magnified picture of the primary reaction product, lead phosphate, is given in Fig. 3. Fig. 4 shows (*a*) the light microscope picture of the final reaction product, cobalt sulfide; (*b*) and (*c*) the x-ray absorption pattern of the primary reaction product, cobalt carbonate; and (*d*) the x-ray absorption picture of the final reaction product in the method for carbonic anhydrase. Fig. 5 *a* shows the x-ray absorption picture of the cobalt-formazan reaction product of the MTT-cobalt method for succinate dehydrogenase and Fig. 5 *b*, the x-ray absorption picture of the Nitro-BT reaction product. Fig. 6 shows (*a*) the light microscope picture and (*b*) the x-ray absorption picture of the reaction product of the thiolacetic acid method.

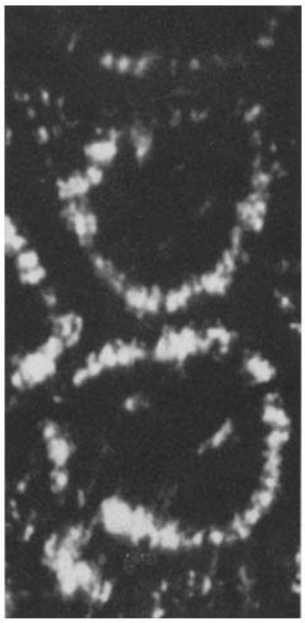


FIGURE 3

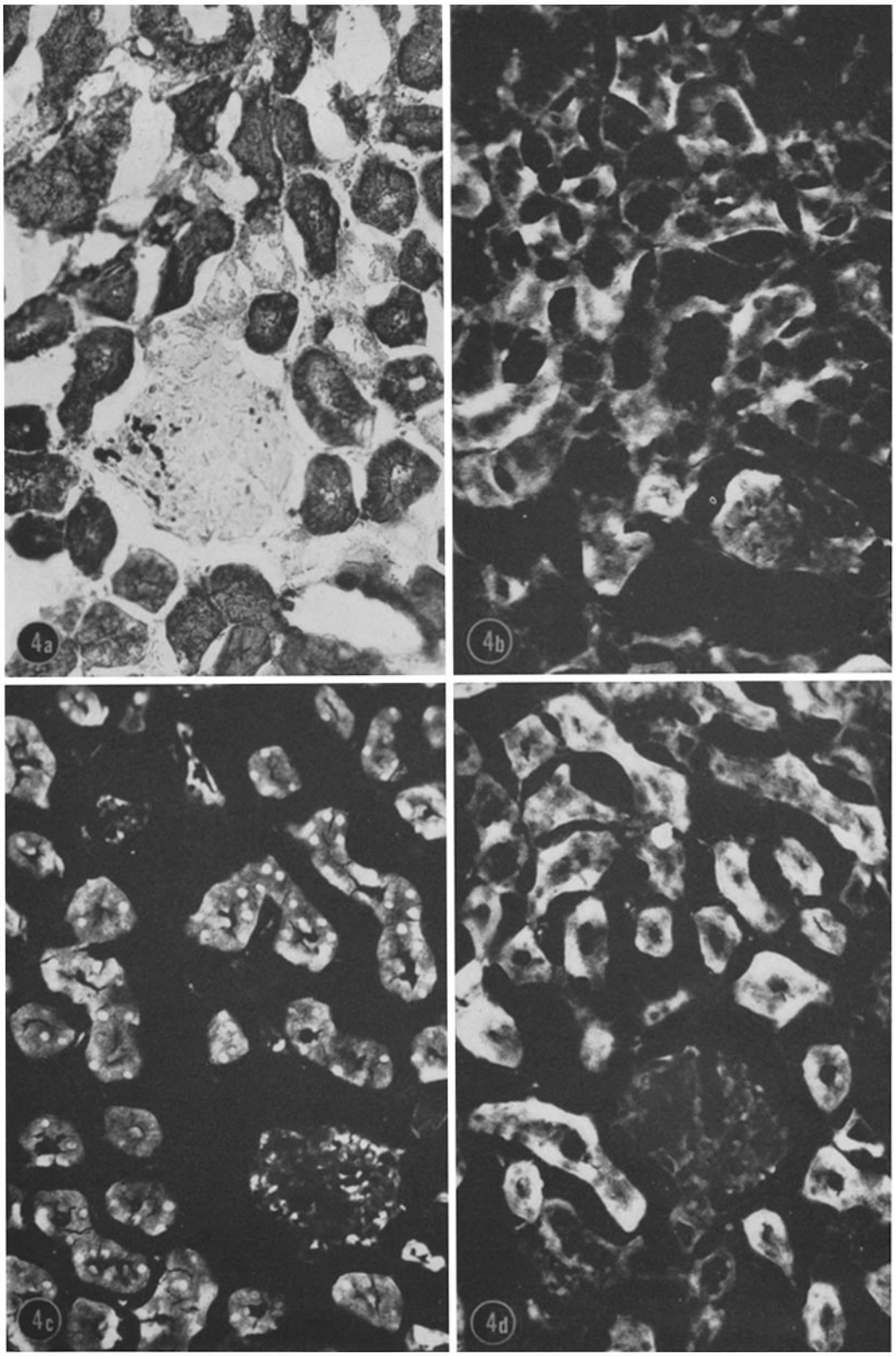
Localisation of the primary reaction product, lead phosphate, in the method for acid phosphatase obtained in the x-ray system. Exposed for 1.5 hours at 1.5 kv and 1.5 mA. × 750.

DISCUSSION

I. *Factors Influencing the Successful Localisation of Reaction Products by Absorption*

(a) Those Inherent in the Reaction Product

(i) PRESENCE OF CERTAIN ELEMENTS. In his detailed consideration of the factors influencing the measurement of elements by historadiography Lindström discusses this factor (30). The possibility of detecting a particular element will depend upon the number of atoms present and the



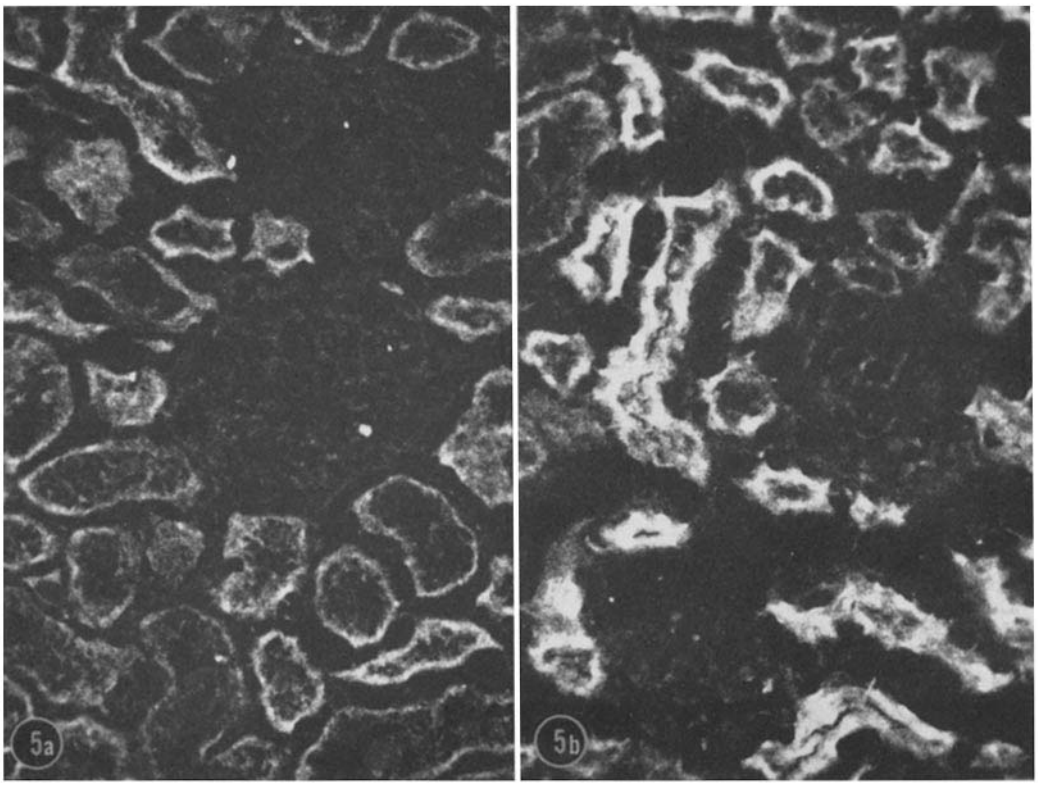


FIGURE 5

Localisation of (a) the cobalt-formazan reaction product of the MTT-cobalt method and (b) the non-chelated reaction product of the Nitro-BT method for succinate dehydrogenase in the x-ray system. Exposed for 1.5 hours at 1.5 kv and 1.5 mA. × 250.

position of its K, L, and M critical absorption edges in the x-ray spectrum. The mass absorption coefficient (μ/ρ) of elements shows a distinct discontinuity at these critical absorption edges and a particular element will absorb heavily on the short wavelength side of that discontinuity.

Inspection of tabulations of the mass absorption coefficients of the elements for the shorter wavelengths up to 2 A (5, 35), and for the longer wavelengths 2.5 to 22.0 A (30), and 8.34 to 44.0 A (23), shows that particular wavelengths can be chosen at which the absorption of x-rays can be attributed mainly to a particular element. It is difficult to determine experimentally the mass absorption coefficient of elements of high atomic number at long wavelengths, and, where computation has been made of their probable absorption coefficients, only elements of low atomic number have been considered. The absorption of x-rays of wavelength 8 A or longer by lead will be considerable but the exact amount still remains to be calculated. The distribution of intensity in the continuum of

FIGURE 4

a, The light microscope picture of the final reaction product for carbonic anhydrase. *b* and *c*, localisation in the x-ray system of the primary reaction product, cobalt carbonate. *d*, the final reaction product, cobalt sulfide. Exposed for 1.5 hours at 1.5 kv and 1.5 mA. × 250.

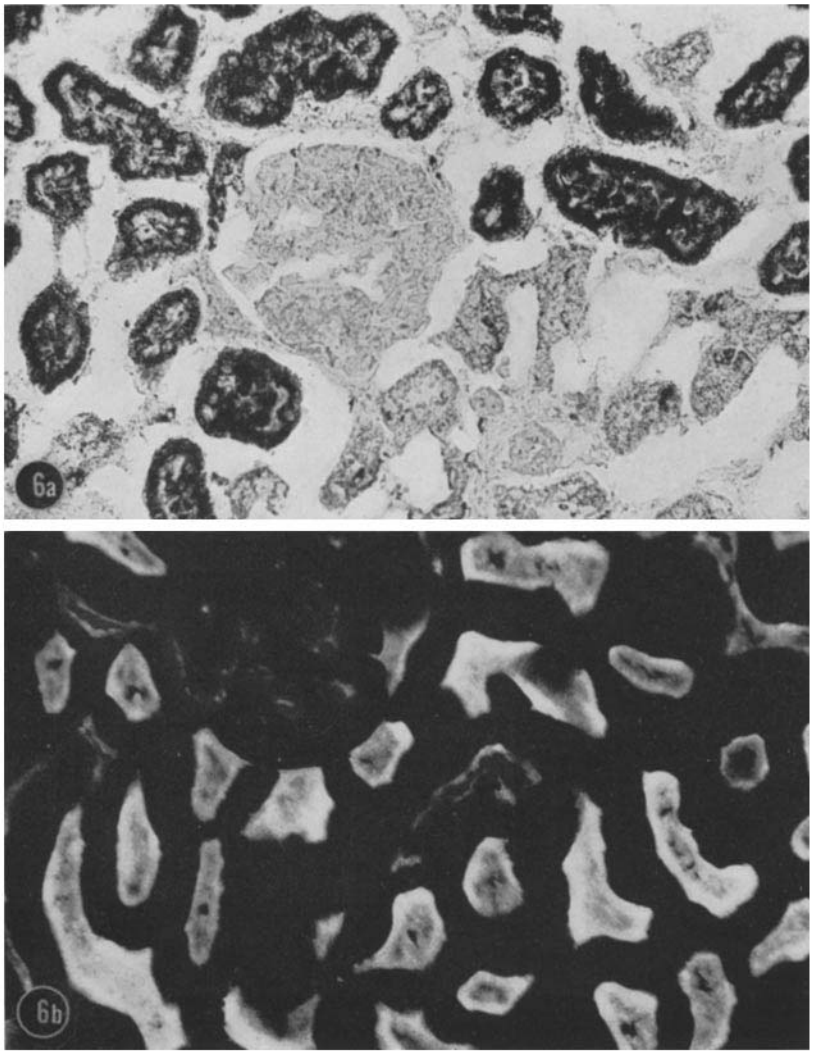


FIGURE 6

Localisation of the reaction product, lead sulfide, for the thiolacetic acid esterase method in (*a*) the light microscope and (*b*) the x-ray system. Exposed for 1.5 hours at 1.5 kv and 1.5 mA. × 250.

x-rays generated at 1.5 kv in the type of apparatus used here is such that 80 per cent lie between 8.25 and 12.5 A (30).

The minimum amount of an element which can be detected has been calculated. It has been shown that the sensitivity of detection is related to the magnitude of the absorption edge; thus the sensitivity is greater for elements of low atomic number (11, 12).

(ii) FORM IN WHICH THE ELEMENT IS PRESENT. The absorption per gram of an absorbing element is given by the mass absorption coefficient, thus the absorption per atom is

$$\mu_a = \frac{\mu}{\rho} \frac{A}{N_0}$$

where μ_a is the atomic absorption coefficient, A is the atomic weight and N_0 is Avogadro's number (6.0247×10^{23} mole $^{-1}$). N_0/A is the number of atoms per gram of absorber.

μ_a is almost completely independent of the

physical and chemical state of the absorbing element. It varies with the atomic number of the absorber and the wavelength of the x-rays.

The mass absorption coefficient of a chemical compound is

$$(\mu/\rho)_c = \Sigma n_i \, (\mu/\rho)_i \, A_i/M$$

where M is the molecular weight, n_i is the number of ith atoms per molecule and A_i is the atomic weight.

Table I shows the mass absorption coefficient at different wavelengths of several compounds which may be deposited as reaction products. It also shows the values for materials commonly found in histological sections, as calculated by Lindström (30).

It can be seen that at the wavelengths commonly used in historadiography (8 to 12 A) the absorption by inorganic compounds is 75 to 180 per cent greater than that of the tissues whereas the organic non-chelated compound has a value virtually the same as the tissues and the organic chelate a value 25 per cent higher.

It has also been shown that one can ignore scattering as a source of error in calculation of the absorption by suitable elements at these wavelengths (25, 30, 35).

The actual amount of absorption per unit mass by a reacting product at a given wavelength will thus depend on

(*a*) The number of atoms of each element deposited.

(*b*) The absorption coefficient of each element deposited.

It will be independent of the chemical combination of the elements.

(iii) MASS PER UNIT AREA OF REACTION PRODUCT. The method of measurement of dry mass developed by Engström and his associates demonstrates that differences of the order of 10 per cent in the mass per unit area of organic matter can be detected when elements of high absorption coefficients at the wavelengths being used are absent.

The contrast obtained at ultra-soft x-ray wavelengths with the reaction products studied here is as much a result of the increase in mass per unit area when the product is deposited as it is of any specific absorption attributable to any one element deposited. The pictures obtained with the Nitro-BT and MTT-cobalt methods illustrate this fact.

This apparent limitation may, in fact, be an advantage since the amount of total reaction product and the amount of cobalt alone could be measured at different wavelengths and thus supply information about the ratio of cobalt/formazan in the reaction product. This is a point of some interest in developing methods for measuring activities of enzymes. It has already been pointed out (2) that the total amount of reaction product deposited in unit time is not a measure of enzyme activity but the total amount of one of the components of it might well be (21).

The amount of reaction product necessary to produce a detectable increase in absorption is probably of the order of 1.0×10^{-12} gm/μ^2 in a section 10 μ thick.

TABLE I

Mass Absorption Coefficients of Compounds at 8.34 A

$Ca_3(PO_4)_2$	1432.0
$Co_3(PO_4)_2$	2076.0
CoS_2	1839.0
Co_2S_3	1993.0
$CoCO_3$	2154.0
$2CoCO_3$-$3Co(OH)_2$	2213.0
half salt Nitro BT	787.4
MTT-Co	1028.0
Animal protein	800.0 at 8 A
DNA	902.0 at 8 A
Collagen	821.0 at 8 A

(iv) PHYSICAL FORM OF REACTION PRODUCT. If the physical form is such that a given amount of reaction product is deposited in a very small volume, then the chances of detecting it are good. It has been pointed out above that absorption coefficients per unit mass have been used as those per unit length would vary with the density of the material. There will thus be a greater possibility of detecting reaction products of high density at small sites of enzyme activity.

(v) SOLUBILITY. The question of solubility and thus diffusion of the reaction product is one which has plagued earlier studies on localisation of enzymes. It has been shown previously for alkaline phosphatase (21) and suggested here for carbonic anhydrase that diffusion is not such a problem with the primary reaction product as it may be with the final one. Study of a primary reaction product is in any case more satisfactory as it dispenses with errors due to incomplete conversion of primary to final reaction product.

(vi) STABILITY OF THE REACTION PROD-

UGT. The reaction product is examined under conditions of high vacuum, high temperature, and quite high energy bombardment. These conditions must be borne in mind in considering the results obtained.

(vii) THICKNESS OF SECTION. For a conventional histological section 2 to 10 μ thick the amount of x-ray absorption will depend on the mass absorption coefficient of the deposited elements and the total dry mass of the compound. If the absorption is due mainly to a particular element, then contrast of the absorption image may be increased by using a thicker section at a shorter wavelength at which the particular element absorbs heavily. The thickness of the section to be used will however depend on the rate of penetration of the substrate in the cytochemical method and the loss of resolution due to absorption at different levels in the section.

It has been pointed out by Cosslett and Nixon (7) that for a given inclusion (and in this case we may consider the reaction product an inclusion in a matrix) maximum contrast is obtained at a suitable wavelength irrespective of whether the inclusion is locally concentrated or dispersed in depth.

(b) Variations Inherent in the Method of Detection

The conditions for the production and detection of x-rays are well known and they have been discussed in some detail in relation to historadiographic techniques (6, 7, 11). In essence they depend on the voltage of the generating equipment, the type of anode material, the filter or other system used to obtain monochromatic or near monochromatic x-rays, and the method of detection. The latter may be photographic or photoelectric. The over-all error in measurement in the historadiographic method is around 5 to 10 per cent for dry weight measurement and 1 to 3 per cent for elementary analysis.

II. *Interpretation of Results*

It has been found that with the x-ray technique the lead phosphate and lead sulfide produced in the method for acid phosphatase both gave a localisation similar to that obtained with the light microscope for lead sulfide. In the method for carbonic anhydrase a similar localisation has been found with the x-ray method and the light microscope when cobalt sulfide was being examined. However, the primary reaction product, cobalt carbonate, has never given a localisation in the x-ray system which corresponds to that for cobalt sulfide. Its localisation is either diffusely spread over all microscopic structures or predominantly in the nuclei with lesser cytoplasmic distribution. It has been suggested by Fand and her associates (14) that the cobalt sulfide localises at sites where zinc is present and gives little if any indication of carbonic anhydrase activity. The present findings suggest that the cobalt sulfide localisation is not a reliable guide to carbonic anhydrase activity but the persistent staining of red blood corpuscles suggests that it might have some specificity. Red blood corpuscles, however, have a high density which gives them a naturally high absorption at the wavelengths used here.

The results for lead sulfide localisation by the x-ray and light microscope methods, in the technique for thiolacetic acid esterase, show excellent correspondence. The heavy absorption in the cytoplasm in the proximal convoluted tubules contrasts with the slight absorption in the brush border.

These results and those obtained previously for alkaline phosphatase (21) show that it is quite easy to visualise the primary, intermediate, and final reaction products of cytochemical reactions which deposit an inorganic salt at the site of enzyme activity. There is no reason why the method cannot be extended to the study of reactions showing similar inorganic products; *e.g.* the methods for specific phosphatases such as 5-nucleotidase, ADP-ase, ATP-ase, and glucose-6-phosphatase (36) or β-glucuronidase (15).

The demonstration of the metal-devoid and metal-containing formazan reaction products of the Nitro-BT and MTT-cobalt methods for succinate dehydrogenase illustrates another application of the method. This approach can be used to study the amount of these reaction products deposited and to attempt to correlate them with the amount of chromophore measured microspectrophotometrically. This approach could also be extended to the metal-diazonium-naphthol complexes introduced by Burstone (3) for identification of esterase and aminopeptidase.

The method is useful in screening cytochemical methods which might prove useful in introducing electron-opaque elements or compounds into tissues to be studied in the electron microscope. The apparatus is cheap (£300/$840) and simple

to use. The method provides a useful bridge in crossing the gap separating colour reaction in the light microscope from electron-contrasting reactions in the electron microscope.

Yet another possibility is that the original idea of Engström and Jakus to apply the method to a study of protein stained with phosphotungstic acid (13) may prove feasible in measuring amino acids by means of metal chelation methods like the Morel-Sisley reaction for tyrosine (16).

Received for publication, May 29, 1962.

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