HISTOCHEMISTRY—A REVIEW[1]

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Histochemistry is presumably as old as histology, since microscopists have always been interested in the nature of the materials that they were examining. As a definitive science, however, histochemistry had its birth, or, shall we say, its baptism with the publication in 1936 of Lison's "Histochimie Animale", in which the author made the first systematic attempt at critical analysis of the techniques and findings up to that date. In the past decade the expansion of the science has been explosive, marked by the publication of five text books or monographs (4, 7, 9, 16, 21),[2] the establishment of a special scientific society, and of a special journal. It is of interest that four of the five books, those dealing exclusively with the analysis of tissue components *in situ*, indicate by their sub-titles an emphasis on the critical or theoretical approach to the subject. The authors are evidently disturbed by the lack of rigor under which the practice in this field has developed in the past and all four of them have almost simultaneously, and in much the same pattern of thought, attempted to remedy this defect. With the leaders in this field selfconsciously critical of their domain it seems not inappropriate to inquire as to the significance of work in histochemistry for the experimental biological sciences.

Let it be said at the outset that no one doubts the validity of the goals of this science. To learn the structural organization of the components of living tissue, and conversely to learn the chemical composition of recognizable tissue structures is a necessary goal of the biological sciences. There is no problem of physiological mechanism that does not in the end come down to the dual question of what goes on (chemically) where (anatomically). There are no doubts as to the worthiness of enterprises directed toward these goals. However, in view of the difficulties and limitations of the techniques of analysis *in situ*, there are legitimate doubts as to whether this is the most fruitful approach to these goals. There are other available techniques in this general domain.

Both in logic and in actual practice there are three separate modes of approach to the central problems of histochemistry: 1) mechanical separation of tissue components, the harvesting in macroscopic yields of tissue components of a given type such as nuclei, mitochondria, etc., and the chemical study of these components by appropriate macroscopic techniques; 2) the isolation of some microscopic tissue segment and its analysis by micro-chemical techniques; 3) the identification of tissue components *in situ*.[3] These three methods of histo-

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[2] Since this manuscript was submitted an additional textbook has been published: Histopathologic Technique and Practical Histochemistry by R. D. Lillie, Blakiston & Co., New York 1954.

[3] The special merits and difficulties of radio-autography will not be discussed in this review.

chemical approach have in the past been largely independent, in the sense that few individuals have worked in more than one of the three fields. Of the five recent books, only that of Glick (7) attempts to survey all three areas. One of the chief reasons for establishing the Histochemical Society and the Journal of Histochemistry and Cytochemistry was to afford workers in these three areas a common forum. Like every scientific method all three of these approaches have each their special limitations. To what extent are they mutually supplementing rather than competing?

A. Mechanical Separation

As Bensley (3) pointed out "to separate separable things before proceeding to their analysis" requires no justifications or defense. By the use of tissue blender and centrifuge it is possible to obtain relatively pure yields of certain morphologically identifiable tissue components. Though special instrumentation may be required for some particular problems much of the work can be done with equipment regularly available in departments of biochemistry and it is naturally this approach which has been most actively exploited by biochemists. It is to be recalled, however, that these methods of separation were developed mainly by anatomists, chiefly by Bensley and his pupils, and that the criteria of purity and homogeneity of the yield have been largely morphological. It is problems connected with the purity and homogeneity of the sample that are still the bugbear of these procedures.

To illustrate both the strengths and weaknesses of this approach the work of Lehninger (14) may be cited. This author found that in a preparation of liver mitochondria the oxidation of certain substrates proceeded without addition of cytochrome C to the incubating fluid, while for other substrates, cytochrome C supplement was required. Lehninger assumed that the mitochondria contained cytochrome C and that those substrates whose dehydrogenases were located in the interior of the mitochondria could be metabolized by these structures without external supplement. For the other substrates he assumed the dehydrogenases were located on the surface of the mitochondria and required cytochrome C in the ambient fluid for their activation. It is plain, however, that the data could be equally well explained by assuming that the preparation included two kinds of mitochondria. Since liver cells in the hepatic and portal regions are not morphologically identical, and since they differ in their pathological behavior in different metabolic diseases, one can reasonably suppose that differences exist in their enzymatic equipment. The assumption that mitochondria from different portions of the liver are identical, or for that matter, the assumption that all the mitochondria of a given cell are identical, is not justified by any positive knowledge. It is, perhaps, a sufficient triumph of the separation technique to be able to decide what enzymes are attached to mitochondria without concluding which are inside and which outside or that all mitochondria are alike. The strengths of this approach lie in the limitless chemical techniques to be applied to the harvest. The weaknesses lie in the uncertainties regarding the purity and homogeneity of the harvests. In the long run,

it would appear that some method of analysis *in situ*, perhaps with visualization by electron microscopy, may be required to resolve the questions of purity and homogeneity of the harvests.

The extent of the difficulties may be made clear by the fact that workers in this field are agreed that cytochrome oxidase is present in mitochondria, and absent in chromosomes,—but whether this enzyme is regularly present or absent in the nucleus is still unresolved.

B. Micro-dissection and Micro-analysis

Whenever feasible the harvesting of macroscopic quantities of the microscopic objects to be analyzed remains the procedure of choice. There are, however, innumerable occasions in biological research when only tiny samples can be obtained for analysis. Advances in micro-analytic techniques during the past generation and especially during the last few years have been phenomenal. The listing in Glick's book (1949) is already partially obsolete. The Carlsberg Laboratory under Linderstrøm-Lang and Holter acquired early and has long maintained a conspicuous lead in this field but many other investigators have contributed brilliantly ingenious advances and refinements. With specially devised pipettes and burettes the sensitivity of ordinary titrations has been increased by a factor of 10^2, with the Cartesian diver the sensitivity of manometric methods has been increased by a factor of 10^3. Balances sensitive to 10^{-9} gm. have been devised. The full limits in combining electronic amplification with micro-chambers and micro-electrodes do not appear to have been reached. No biological laboratory can afford to be unaware of the possible utility of these ingenious and sometimes exquisitely simple gadgets. In very many instances the sensitivity of the analytic procedure exceeds the reliability of dissection of the sample to be analyzed. The nucleus cannot be dissected out of a single cell more free from cytoplasmic contamination than those millions of nuclei isolated from a homogenate by differential centrifugation. As a matter of fact the smallest mammalian tissue samples that it is convenient to handle usually are composed of thousands or tens of thousands of cells.

Faced by the difficulties of reliable micro-dissection Linderstrøm-Lang and Mogensen (15) devised the ingenious procedure of alternate histological and biochemical sampling. In the classical application of this technique to the analysis of the gastric mucosa, the stomach wall was frozen and a small cylinder of tissue bored out. Serial sections of this cylinder were cut on the freezing microtome and alternating sections used for chemical and histological examination. Correlations were made between the chemical findings and cell counts of the various cell types present in the adjacent sections. Pepsinogen content of the sections was found to rise and fall with the counts of chief cells and the conclusion was justified that pepsinogen must be located in the neighborhood of the chief cells. Since there are no other structures regularly found in the neighborhood of the chief cells which would be likely receptacles for this enzyme, it was entirely reasonable to conclude that the locus of pepsinogen is within the chief cells.

The limits of reliability of the sampling technique can be seen in another aspect of this study. Chloride content of the sections was found to be positively correlated with the number of parietal cells. One might be inclined to conclude that the parietal cells were, therefore, the loci of hydrochloric acid secretion. However, Lison (18) using a method of analysis *in situ* for chloride found the main deposits of his reaction product in the connective tissue spaces. Moreover, the volume of connective tissue spaces in the sections happened to be positively correlated with parietal cell number.

If the parietal cells are the site of hydrochloric acid secretion then one would reasonably expect to find chloride in their intracellular canaliculi. The volume of these tiny channels, approximately 1–2 microns in diameter, is so small that the amount of chloride which they contain would be negligible compared to random experimental variables in the Linderstrøm-Lang technique unless the concentration of chloride in the intracanalicular fluid were very high. Since the chloride concentration of gastric secretion is very nearly the same as that of extracellular fluid significant results could hardly be expected by the technique used. On the other hand, Lison is himself well aware that *in situ* localization of chloride by his method is reliable only to about 20 microns. Consequently, the failure of Lison to find chloride in the parietal cell canaliculi does not prove that these cells are not the source of acid secretion.

Within the limitations of the argument as outlined above the technique of alternate histological and chemical assay is a powerful tool for the histochemical analysis of tissues of stratified structure. Bahn and Glick (2) have applied the method with excellent results in a study of the adrenal glands. The strata must, however, be wide compared to the thickness of histologic sections. Anfinsen *et al.* (1) attempted to modify the method so that chemical and histologic assay could be made on the same sections. They applied this technique to the localization of cholinesterase in the retina. Though in principle the method is unobjectionable, in practice the limits of accuracy in localization were substantial since sections could not be cut absolutely parallel to the retinal surface and hence did not represent histologically homogeneous samples. Subsequent studies by Koelle and Friedenwald (13) by a technique of analysis *in situ* revealed a localization of cholinesterase differing from that found by Anfinsen by about 20 microns. There is evidence that the localization *in situ* by the technique used is reliable to within about 2 microns.

If we compare the two approaches to histochemistry that have been so far discussed, it must be clear that whenever macroscopic quantities of the material of interest can be obtained by mechanical separation the unlimited freedom of chemical procedure makes this the first choice. There are, however, vast regions of biological interest where the materials to be studied cannot be harvested in macroscopic lots. The Waring blender and centrifuge are not adapted for separating chief cells and parietal cells from the gastric mucosa, nor would there be much interest in the analysis of a mixed sample of mitochondria from the whole population of gastric mucosal cells. Techniques of micro-analysis when available extend the area of study to the limits of mechanical micro-dissection,

and the device of alternative histologic and chemical assay extends the analysis beyond the limits of mechanical micro-dissection in those particular organs in which structural stratification makes this approach feasible. Just as the macrochemical approach has appealed primarily to biochemists, so the technique of micro-dissection and micro-analysis has appealed primarily to physiologists and experimental embryologists.

C. Analysis in Situ

There are, however, tissues whose structural complexity does not allow analysis by strata. There are structures of interest too small to be separated by macro- or micro-dissection. Surely, for instance, the stratification of enzymes in kidney tubule cells is of interest in respect to secretory activity. Which enzymes are near the basal boundary, which near the brush border, which diffuse in the cytoplasm, which in the nucleus? These questions are quite inaccessible by the techniques and approaches so far considered. Only under the microscope can these loci of interest be clearly distinguished.

To use the microscope both as a chemical analyzer and as a localizer places both aspects of the procedure under severe handicap. Only those chemical procedures are applicable which result in an insoluble and optically recognizable (colored or fluorescent) reaction product. Suitable chemical reactions of adequate specificity and sensitivity are limited in number. Chemical reagents and reactions which destroy the tissue or disorganize its structure must be avoided. Special conditions must be imposed to limit diffusion of reactants or reaction product and special tests and controls are required to decide whether or not, and to what degree, limitation of diffusion has been successful.

No histochemical reaction is absolutely specific, limitlessly sensitive, localizable with absolute precision. For each procedure the limits of specificity, sensitivity and localization need to be worked out, but the criteria of these analyses are not simple nor precise. However, even though the criteria of precision are themselves imprecise, decisions within reasonable limits can be reached which extend the reliability of localization by a factor of 10 or 100 beyond that achieved by the separation or sampling techniques.

Though problems of specificity, sensitivity and localization are involved in all instances of analysis *in situ* they are of such varying weight in different circumstances that two extremes need to be considered separately.

a. Fixed tissue components. By "fixed tissue" what is usually meant is "fixable" tissue and the problem of reliability of localization of these components resolves itself into the problem of the reliability of fixation. There are no precise criteria of the adequacy of fixation but the recognition and analysis of fixation artefacts is as elementary a procedure for the morphologist as the weighing of a sample is to a chemist. Since a fixative must, as a rule, penetrate the tissue block from the outside, a time and concentration gradient of fixation is established and fixation artefacts can be produced for study as desired. How well the morphologist performed his analysis of fixation artefacts and choice of fixation procedures may be gauged by recent findings with electron microscopy.

At any given level of microscopic resolution some uncertainty necessarily affects judgment concerning the presence or absence of fixation artefacts at the very limit of resolution, but a study at higher magnification can give very firm answers regarding the presence or absence of fixation artefacts affecting the picture at lower magnifications. Electron microscopy, while afflicted with its own doubts and perplexities, suffices to resolve those of the light microscope. Fixation procedures which light microscopists already thought were optimal can now be given a clean bill of health as a result of electron microscope studies.

The best fixative from the viewpoint of morphology appears to be osmic acid. This is not often the most convenient fixative for histochemical studies but the morphologist once provided with a satisfactory fixation can work his way upwards through less satisfactory fixations determining at each level the limits of reliability. The morphologist is, therefore, able to state within what optical resolution his fixation is reliable. He rarely makes such a numerical estimate of his probable error of localization because the adequacy of fixation may vary from place to place in his sections and he tries to pick the best fixed regions for his judgments.

The technique of rapid freezing and subsequent dehydration at low temperatures (−30°C.), though not, strictly speaking, a fixation method is, nevertheless, a way of rapidly immobilizing tissue components. Depending on the rate of cooling and other factors, ice crystals form in the tissue which reveal themselves in subsequently prepared sections as cracks and clefts in the histologic picture. Under optimal conditions of preparation such cracks are not visible with the light microscope, but electron microscopy of such preparations reveals ice clefts often of about 0.1 micron breadth. The immobilization by freezing drying is therefore subject to error of the order of ±0.1 micron. Since this is below the resolution of the light microscope this error is negligible in light microscopy. This does not mean, of course, that subsequent steps in the histological procedure may not introduce errors of a much larger order.

Chemical identification of fixed tissue components involves the development of some color reaction under conditions such that the test substance and its colored reaction product remain insoluble. This is not as difficult as it would seem, for very often the test substance or its reaction product, though soluble in a pure state, is so enmeshed in the protein coagulum of the cell as to resist extraction even on severe test. From this it is plain that by prolonging the steps in the histochemical procedure and multiplying the washings one can test quite stringently the solubility of the critical substances. It should be pointed out that the conditions of histologic technique utilize enormous volumes of solvent relative to the precipitate. A precipitate that is not diminished in this process cannot have crept far from its initial position.

Criteria of specificity are similar to those in qualitative chemistry in general, but the environment of the test substance is less controllable and anomalies of reaction are not too rare. Specificity of identification can be very much enhanced if two or more different reactions can be applied and compared. Sometimes specific blocking reactions can be particularly helpful. All of these aspects

have their parallels in test-tube chemistry and do not need to be elaborated here.

Analysis *in situ* is a non-quantitative procedure. Sensitivity, therefore, merely concerns the limits at which the reaction is discernible. Since under the microscope a colored layer only a few microns thick is to be inspected, the light absorption of the reaction product needs to be very intense. Colors which appear brilliant in the test-tube may be unrecognizably faint in the section. The test substance must also be dispersed in particles that are beyond microscopic resolution, though a heap of individually sub-microscopic particles may be visible if the particles are close enough together to yield an appreciable net light absorption.

In respect to the histochemistry of fixed tissue components the problems of localization are, so to speak, under satisfactory control. Limitations are imposed mainly by the small number of substances that may be tested for. Work in this field has been pursued mainly by histologists since it is a direct outgrowth of their studies in pure morphology. Often the questions easiest to answer are of little interest to the physiologist or pharmacologist. What does the latter care whether the Golgi apparatus contains mucoids or not, though he will surely not be able to speculate fruitfully over the function of the Golgi apparatus until its composition and structure are better known. On the other hand, a systematic and comprehensive study of fixed tissue histochemistry such as that by Gersh and Baker (6) on the thyroid, becomes automatically the basis for physiological and pharmacological questionings and even a tool for some aspects of such studies.

b. Diffusible components. The difficulties in the localization of diffusible components may be exemplified in the still unsolved problems in respect to sodium ion and chloride ion. There is no suggestion of a feasible method for localizing sodium ion. The best method for chloride ion (5) has many loopholes. In brief, the method consists in rapid freezing of the tissue, drying in vacuum (preferably at −60°C.), embedding in paraffin, sectioning, mounting, etc., and precipitating the chloride ion as silver chloride by immersing the section in a concentrated solution of silver nitrate. The silver salt is then reduced to metal as in photography. From the dehydration to the final immersion, maximal precautions are employed to avoid water vapor or any solvent for chloride salts. Owing to the freezing and drying, the tissue proteins must then be present as a lattice, rather than a film, with ultramicroscopic crystals of sodium chloride (and other substances) scattered through the lattice. This structure is now flooded with concentrated silver nitrate solution. The chloride must dissolve before it is precipitated by the silver. The protein lattice must be hydrated before it is salted out. Under conditions of saturation the smallest silver chloride crystals must dissolve and the large ones grow. Some movement of the chloride must therefore occur in these procedures. Subsequent reduction of the silver ion to metal is probably above criticism. Judging by the distribution of silver granules at the boundary of tissues known to be almost free of chlorides, for instance muscle fibers, the range of diffusion is about 5 microns and this would

be a rough measure of the limits of accuracy of localization. The method will not answer the important question of whether or not chloride is present in the cytoplasm of a capillary endothelial cell. It will not tell where hydrochloric acid is secreted in the stomach, since hydrochloric acid, if solidified in the freezing process, will evaporate along with water vapor during the dehydration.

c. Enzyme localization. The problem of diffusion artefacts comes up in particularly aggravated form in respect to enzyme localization since, in this case, one must be concerned with possible diffusion of both enzyme and reaction products. The diffusion of enzymes is so markedly influenced by the technique of pre-treatment of the tissue that these two matters must be discussed together.

The present reviewer has long felt that there were obvious advantages in avoiding unnecessary denaturation of the enzymes before looking for them and, consequently, has attempted enzyme localization chiefly in fresh tissue slices or fresh frozen tissue sections. These procedures are not without their special difficulties. In the undisturbed fresh tissue negative results may be due to failure of penetration. In fresh frozen sections, not only is diffusion of enzymes at a maximum, but the slimy cytoplasm of freshly cut cells tends to run so that the histological pattern may be disturbed. In a good many instances high concentrations of some indifferent salt in the incubating solution help to give the section more rigidity and to limit enzyme diffusion. Maengwyn-Davies and Friedenwald (17) tested for enzyme diffusion by seeing whether or not activity could be washed out of the sections and recovered in the wash fluid. Seligman *et al.* (24) have placed this method on a semi-quantitative basis. Most of the substrate specific alkaline phosphatases so far recognized were demonstrated in this way to be desmo-enzymes, but a cofactor of the enzyme acting on fructose 1,6-diphosphate was found readily extractable from the tissues (17). Satisfactory histological localization of this enzyme was achieved in the presence of half saturated sodium acetate.

Seligman *et al.* (22) have recently shown that fixation in cold 4 per cent formalin leaves the activity of many enzymes relatively undisturbed. When this procedure is permissible it can greatly diminish the technical nuisances of fresh frozen sections. Gomori (9) prefers, when possible, to fix his tissues in cold alcohol or acetone and embed in paraffin. There are enzymes that survive these procedures but whether or not survival is influenced by the immediate cellular environment of the enzyme is not known. If it is, then the residual pattern of distribution of the surviving enzyme may give a false representation of that natively present.

In respect to alkaline phosphatases comparisons have been made of frozen section and embedded section technique. The results are extremely complex. In the kidney, alkaline phosphatase activity for glycerophosphate in the fresh state is found in desmo-enzymes. There are two such enzymes capable of hydrolyzing glycerophosphate (11, 17), distinguishable by their reaction to specific activators and inhibitors and differing in their histological distribution. These two enzymes show differing but, in any case, highly restricted substrate speci-

ficity. Acetone powders of kidney contain a soluble alkaline phosphatase which is extremely unselective in its substrate requirement. The histologic pattern of activity for glycerophosphate found in acetone-fixed material does not agree with that of either of the two enzymes present in fresh tissue but resembles most closely that found in fresh tissue with an aryl-phosphate as substrate.

Since a water soluble phosphatase is present in acetone powders, the question of enzyme diffusion in the Gomori technique is a real one. Gomori (8) inactivated the phosphatase in some tissue sections and then exposed the sections to a concentrated solution of the enzyme. After appropriate washing the location of the adsorbed enzyme was tested by the usual procedure. It was found to differ grossly from that normally found in active tissues. Martin and Jacoby (19) using the Gomori technique placed an inactivated section on a slide and on top of this an active section. Reaction product was found in the inactivated section in regions closest to the most active loci in the overlying section. In order to distinguish between possible diffusion of enzyme and of reaction product (see below) these authors repeated the experiment covering the inactivated section with a film of collodion. Since this did not prevent staining of the inactive section they concluded that diffusion of reaction product, but not of enzyme, was present. It may be seen from this discussion that criteria for the presence or absence of enzyme diffusion are not lacking.

As a compromise between fresh frozen sections and the Gomori technique Danielli (4) recommends freezing drying with paraffin embedding. Pearse (21) points out that freezing drying is not fixation and that many tissue components preserve their water solubility unchanged. Exposure to hot paraffin has, however, been shown to inactivate more than half of the alkaline phosphatase. The histologic pattern obtained is similar to that with the Gomori technique. In experiments similar to those of Martin and Jacoby, Danielli concluded there was no enzyme diffusion and also no diffusion of reaction product!

d. Diffusion of reaction product. Nothing is absolutely insoluble. As a matter of fact, most enzymatic histochemical procedures operate under circumstances in which the solubility of the reaction product is 10^{-3} to 10^{-5} M. In the neighborhood of the enzyme there must be a zone of saturation. Within this zone precipitates can be expected to form depending, on the one hand, on the degree of supersaturation, and, on the other hand, on the existence of local factors, *e.g.*, surface charges, adsorptive forces, etc. which promote nucleus formation. The formation and growth of these precipitates cannot be limited absolutely to the loci of the enzyme source and must be influenced to some degree by the pattern of distribution of adsorbing loci, etc. The question therefore, is not whether diffusion of reaction product exists, but how we can measure it, how we can limit it, how we can distinguish nonspecific tissue staining from enzyme localization.

Johansen and Linderstrøm-Lang (12) have attempted a theoretical analysis of the precipitation of phosphate in the Gomori alkaline phosphatase reaction which led them to the conclusion that diffusion in this case must be so extreme

that precipitation is determined almost exclusively by adsorptive forces, etc., and that the reliability of enzyme localization is almost nil. Their argument may be summarized as follows: phosphate is presumed to be manufactured within sources, *i.e.*, enzyme loci defined as spheres of 100 Å radius which are arranged in any arbitrary pattern within the cell, this pattern being repeated in adjacent cells. Assuming simultaneous access of substrate to all enzyme loci, phosphate concentrations should rise symmetrically in all cells and no net diffusion across cell boundaries need be considered. The cell wall, defined as a sphere of 10 microns diameter, can therefore be considered as an impermeable barrier. With this ingenious assumption the diffusion equation is integrable, and an expression is obtained for the maximum concentration difference within the cell, *i.e.*, the concentration difference between source and cell boundary as a function of time. The parameters of this equation are the turnover rate of the enzyme, the diffusion rate of phosphate, and the pattern of agglomeration of enzyme sources into clusters. Setting not unreasonable estimates for these parameters the answer is obtained that, except for the first brief instant, the relative excess of concentration at the source is negligibly small. Moreover, it is argued, calcium phosphate complexes tend to form supersaturated solutions and sharply localized precipitations could be expected only if the concentration gradient were steep.

In defending the histochemical method against this attack, Gomori and Benditt (10) disputed the tendency to form supersaturated solutions and showed that a concentration of phosphate of 10^{-5} M sufficed to cause prompt precipitation in the standard incubating solution for alkaline phosphatase. They argue, moreover, that the figure used by Johansen and Linderstrøm-Lang for estimating enzyme activity per cell could be wrong by a factor of 10^3 and that diffusion in tissues may be slower than in water. It might be added that, in the presence of a large excess of calcium, most of the phosphate in solution would probably exist as some sort of calcium phosphate complex, not as free $HPO_4^=$, and that the diffusion rate of phosphate ions in water is of little relevance. This would certainly be true in supersaturated solutions envisaged by the Johansen and Linderstrøm-Lang theory. Moreover, if foci of potential adsorption, nucleus formation, etc., are distributed sufficiently densely in the tissue, the argument of Johansen and Linderstrøm-Lang even if undisputably correct on other scores would merely serve to point out the grainyness of the localization analogous to that of a photographic emulsion.

There is, however, no need to accept the argument as even theoretically sound. The particulate nature of ions makes it possible, using the Johansen and Linderstrøm-Lang assumptions, to reach precisely opposite conclusions. According to these assumptions there exist in cells loci of potential adsorption and nucleus formation so that the concentration of phosphate at the cell boundary should not in the model rise much above precipitation level (approximately 10^{-5} M). The question is, how much higher than this can the concentration rise in the "source". Using Avogadro's number, 10^{-5} M means 6×10^{18} phosphate ions per liter. The assumed source according to the theory has a volume of 4000 μm^3

or 4×10^{-20} liter. At the precipitating concentration, then, there will be on the average, a small fraction of one phosphate ion per source. The appearance of one new phosphate ion within the source will increase the concentration ratio from source to boundary not by a small percentage as calculated on diffusion theory but many fold. According to the assumptions of the theory the conditions for highly precise localization of the source are indeed excellent. The conclusion of this paradoxical argument is not that diffusion of reaction product does not exist but that estimation of its influence on the microscopic picture by macroscopic diffusion theory is hazardous.

It would be possible, no doubt, to develop an appropriate theory of molecular diffusion applicable to this problem, but, since the parameters of such a theory are unknown, mathematical virtuosity on this point would be wasted. We are left with the need to consider the direct evidence of the histochemical preparations themselves. The most critical relevant work has been done on the alkaline phosphatases.

In a detailed study (17) the results of using different substrates have been compared. On fresh frozen tissue sections consistent and reproducible differences in the reaction pattern were demonstrable with certain different substrates. In each case the reaction product identified (phosphate) was the same and the mode of precipitation and visualization was the same. If the pattern were determined solely by loci of potential nucleus formation for calcium phosphate no such differences were to be expected. With some substrates, the reaction product was in the cortical tubular cells of the kidney, with others, in the vascular endothelium and glomeruli. With some, the deposit was nuclear, with others, exclusively cytoplasmic; with some, it was at the brush border, with others, at the basal region of the cells. From these differences in localization, in addition to other differences in response to activators or inhibitors, the existence of a number of substrate-specific alkaline phosphatases was concluded and some of these differentiations have been subsequently confirmed by *in vitro* studies (11), but it may also be concluded that the sources of the reaction product, that is the loci of the distinguishable enzymes, differ from one another. A resolving power of 2–3 microns would suffice to make the distinctions that were made, and we must conclude that the reliability of the localization is at least that good.

In the case of naphthol phosphate the phosphatase localization can be tested by two independent methods; that of Gomori for phosphate and that of Manheimer and Seligman (18) for naphthol. After enzymatic hydrolysis the location of the two reaction products is the same. It is not to be expected that loci of potential nucleus formation would be the same for naphthol as for calcium phosphate complexes, but this can be separately tested. Seligman *et al.* (23, 24) have utilized naphthol esters as substrates for esterase, sulfatase, and glucuronidase, localizing the reaction product naphthol by the same procedure in each case. The patterns for these different enzymes differ from one another and from that of the aryl-phosphatase.

Gomori (8) has specifically investigated the localization of calcium phosphate in tissues independent of local enzymatic action. He inactivated some sections

and placed them close to other active sections in the incubation solution. The inactive sections became stained but the pattern was different from that normally seen in active sections. Similar experiments were also performed by Novikoff (20) and by Martin and Jacoby (19) with similar results. As a matter of fact, active sections are not required to saturate the solution with phosphate. Glycerophosphate can be made to hydrolyze spontaneously with hydrogen peroxide according to Danielli (4), or the substrate can be omitted from the solution and enough inorganic phosphate added to form a cloud. In the latter case, the enymes in the tissue need not be inactivated and a more perfect experimental control is thus achieved.

It is of some interest to consider the results of incubating tissue sections in solutions, saturated with calcium phosphate in the presence of excess calcium, with subsequent development of the color reaction for deposited calcium. Fresh frozen sections may be incubated for many hours without any visible stain resulting. If kept in the solution 24 hours or more a faint diffuse stain results. On examination with the highest resolution of the light microscope individual grains of deposit are not recognizable. Both the grain size and grain separation must be smaller than the limits of light microscopy. When the same experiment is done on fixed sections the results are much the same, but in addition to the diffuse cytoplasmic stain there is now an intense nuclear stain. Those familiar with the well known differences in stainability of fixed and unfixed cell nuclei will not be surprised by this result. Danielli (4) using frozen dried unfixed sections failed to obtain nuclear staining in experiments like those of Novikoff. Irregular nuclear staining is seen in fresh frozen sections incubated for several days. Whether this is due to changes in the staining capacity of the nuclei or to the operation of nucleic acid phosphatase on endogenous substrate is not clear.

An appropriate technique for sharpening the enzyme localization and, at the same time, eliciting non-specific staining of controls is to saturate the experimental incubating solution with reaction product, and to use a similarly saturated solution either without substrate or with an inhibitor for control. Under these circumstances the differences between experimental sections and controls can be properly attributed to the enzyme.

What is plain from these experiments is that appropriate controls are possible in order to distinguish artefactual tissue staining from enzyme localization. Moreover, the excessively fine grain of these staining deposits shows that for calcium phosphate there exist in the tissue potential loci of nucleus formation so densely distributed as to be beyond the resolution of the light microscope. One can well imagine that every accessible acid and basic group in the tissue protein may in fact be such a potential locus.

When the phosphatase reaction is performed on active tissue sections a black granular deposit results differing from the diffuse grey brown stain. Under optimal conditions in respect to the crispness of the resulting histologic picture the individual granules are less than a micron in diameter but still not beyond the resolution of the light microscope. Between them the tissue appears un-

stained. This is true even when the incubation fluid has been deliberately saturated with phosphate before the tissue sections were placed in it. It seems likely that in the neighborhood of larger crystals the smallest ones dissolve and the larger ones grow. Ample opportunity for this to occur exists in the routine color development steps.

With adenosinetriphosphate as substrate and short incubation most tissues showed a very intense stain in the capillary endothelium with little if any stain of other tissue components. Close examination of these cells, however, shows that the granular deposit does not stop sharply at the cell boundary but that isolated granules lie outside the cell, the number of these granules decreasing rapidly with increasing distance from the cell wall. Since this same picture is seen in all tissues irrespective of the nature of the cells adjoining the capillaries and even when the capillaries lie in acellular interstitial tissue it is reasonable to suppose that the granules are the result of enzyme activity within the endothelial cell. On this assumption one can use the distribution of the extravascular granules as a means to estimate the marksmanship of the histochemical localization. The standard deviation of the granule from its source would appear in this instance to be of the order of 0.5 microns. Sources separated by 2 microns ($3\sqrt{2}$ S.D.) should, under favorable circumstances, be resolvable.

Resolution of this degree has, so far, been established only for the alkaline phosphatases (on fresh frozen tissue) and thiocholinesterases. Whether improvements in technique can lead to still higher resolution is uncertain, but the possibility of knowledge approaching this level of precision is surely sufficient to excite the biological investigator.

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