cally for succinic dehydrogenase. These results will be described in detail elsewhere. Thick sections of heart, kidney, liver, and brain stained consistently, although with variable intensity among the species studied. This is in accord with the findings of others using *in vitro* techniques (*6–8*). The tissues with the greatest succinic dehydrogenase activity (aerobic) are shown in Fig. 2 (1–5). Many tissues showed no evidence of succinic dehydrogenase activity under these conditions aerobically. Some of these same tissues exhibited activity under anaerobic conditions (Table 1). Sections of tissues heavily contaminated with bacteria, such as small intestine and colon, failed to reduce BT. It is therefore unlikely that enzymatic activity associated with 2 hr of bacterial growth was responsible for the results obtained.

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The Validity of Histochemical Phosphatase Methods on the Intracellular Level

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We have recently found that nuclei isolated from rat liver cells contain only a small fraction of the total alkaline phosphatase activity (*1*). This low activity is in sharp contrast to the intense staining of nuclei in sectioned material prepared by the method of Gomori (*2*) and Takamatsu (*3*). Since we could find no evidence of enzyme extraction from the nuclei during our isolation procedure (*4*), we have investigated the possibility that calcium phosphate and/or enzyme was preferentially adsorbed by the nuclei in the course of the histochemical procedure. The results have led us to conclude that the Gomori-Takamatsu technique cannot be relied on to indicate intracellular enzyme sites.

Alkaline Phosphatase Activity by Gomori Method

The procedure used is that of Gomori (*5*). Thin slices of tissue are fixed in ice-cold acetone. Sections, cut at 7 μ, are incubated in the substrate medium containg $1.7 \times 10^{-2} M$ Na glycerophosphate (52% α), $2.3 \times 10^{-2} M$ sodium diethyl barbiturate, $3.1 \times 10^{-3} M$ $MgSO_4$, and $8.4 \times 10^{-3} M$ $CaCl_2$, adjusted to pH 9.4. Incubation is at 37.5°. The sites of calcium phosphate deposition are visualized by treatment first with cobalt nitrate and then with ammonium polysulfide. The same incubation medium is used for "squashes" of *Drosophila* salivary glands prepared in salt solution according to Krugelis (*6*) and fixed in 95% alcohol for 3–48 hr.

Rat liver. Typical results following long incubation (17 hr) are shown in Figs. 1 and 3. Intense stain is seen in the endothelium and lumen of the blood capillaries surrounding the bile ducts. The low-power view shows that the nuclei, cytoplasm, and bile canaliculi stain more darkly in the hepatic cells of the periportal areas than in those surrounding the central veins. Under high power, the dark staining of nucleoli, chromatin, and nuclear membrane is evident.

After short incubation periods (30 min) the periductal capillaries and certain of the sinusoidal cells stain intensely (Fig. 6). The only nuclei which stain are those of these sinusoidal cells and of the bile duct cells adjacent to the darkly stained capillaries. The hepatic cell nuclei are no darker than those of control slides incubated in the Gomori medium from which substrate is omitted.

Guinea pig kidney. The kidneys used are surrounded by a connective tissue capsule formed in response to wrapping with cellophane (*7*). After incubation of the slides in the glycerophosphate medium for 30 min, the brush borders in the proximal convoluted tubules stain intensely black. The nuclei of these tubules are quite dark; those of the distal tubules (lacking brush borders) are only lightly stained. After incubation for 17 hr (Fig. 7) there is a widespread darkening of the cells of the proximal convoluted tubules, and their nuclei are intensely stained. The nuclei of the distal tubules are much lighter. In the perirenal connective tissue capsule, a gradient in staining of fibroblast nuclei is apparent, with the stain fairly intense in the layer adjacent to the dark kidney cortex and absent in the layers furthest removed from the kidney tissue (*7*). Fig. 7 illustrates what may be found occasionally: a fairly dark staining of the nuclei of the distal tubules and perirenal capsule which lie near the dark proximal tubules and a much lighter staining of the nuclei further removed from the proximal tubules.

Drosophila salivary glands. As demonstrated by Krugelis (*6*) and Danielli and Catcheside (*8*), the Feulgen-positive chromosomal bands stain intensely (Fig. 9). The cytoplasm of intact cells stains, sometimes quite deeply (*cf.* Fig. 1 in Krugelis [*6*]). An apparent spread of stain from some darkly staining material present in the "squashes" to the chromosomes of a salivary nucleus is sometimes evident; the bands are visible in the darkened ends of the chromosomes but not at the other ends.

Calcium Phosphate Adsorption

Identical staining may be obtained in the nucleoli and chromatin of rat liver and guinea pig kidney and

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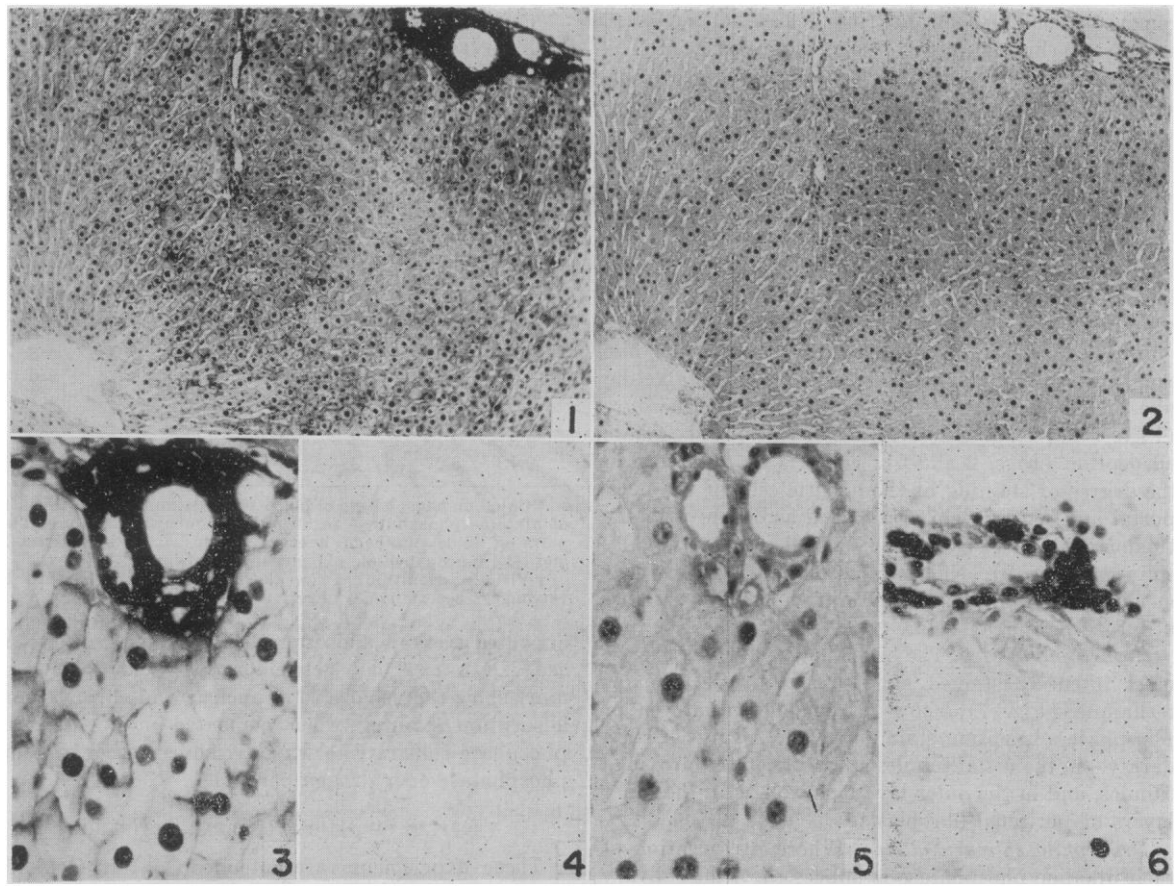


FIG. 1. Rat liver (×85). Standard Gomori procedure. Incubation time: 17 hr.
FIG. 2. Following section (×85), inactivated and incubated for 16 hr in substrate medium to which was added supernatant fluid containing 9 μ *M*-units of alkaline phosphatase activity.
FIG. 3. Another area of section shown in Fig. 1 (×400).
FIG. 4. Same area from inactivated section incubated in standard Gomori substrate medium for 17 hr (×400).
FIG. 5. Same area from section shown in Fig. 2 (×400).
FIG. 6. Rat liver (×385). Standard Gomori procedure. Incubation time: 30 min.

in the bands of *Drosophila* salivary chromosomes even when these structures are deprived of any inherent enzyme they may possess. All alkaline phosphatase of the sections or "squashes" is inactivated by treatment with distilled water at 90° C for 2 min or more, so that even prolonged incubation in the standard substrate medium fails to produce stain anywhere on the slide (Fig. 4). Nuclear and chromosomal staining may, however, be obtained on these inactivated slides if phosphate is split from the substrate of the medium to produce a precipitate of calcium phosphate. The phosphate liberation can be achieved enzymatically, through the addition of supernatant fluid obtained by differential centrifugation of rat liver or of purified alkaline phosphatase solution,[2] or nonenzymatically by the addition of dilute hydrogen peroxide (*10*).

The possibility that the added phosphatase produced the nuclear staining directly, by splitting phosphate from the nucleic acids of the chromatin and nucleoli, is excluded by three different experiments: (1) Sections (both normal and inactivated) are incubated in media containing all ingredients *except substrate,* but to which alkaline phosphatase solution is added. In the absence of phosphate ester, calcium phosphate precipitate is not formed, and the sections remain unstained when treated with sulfide. (2) Sections incubated in media containing alkaline phosphatase, with or without substrate, show no decrease in methyl-green staining intensity (measured microspectrophotometrically) as compared with control sections not in contact with phosphatase. If, as is generally considered (*11*), methyl green combines stoichiometrically with polymerized desoxyribonucleic acid (DNA) through the latter's phosphate residues the unchanged staining capacity of the nuclei would indicate that those residues which participate in the combination with the dye are not split by the phosphatase. There seems no reason, a priori, to believe that the same is not true of the other phosphate groups. (3) Nucleic acids are

[2] Most of the alkaline phosphatase used in these experiments was prepared from calf intestine (*9*); it split about 900 μ *M* of ester (disodium phenyl phosphate or sodium β glycerophosphate)/ml/hr. In a few experiments a more highly purified solution, free of tryptic activity, was used; it split about 8,400 μ *M*/ml/hr.

removed from nucleoli by ribonuclease (RNA-ase)[3] and from chromatin by desoxyribonuclease (DNA-ase),[4] and the sections, or "squashes," are then inactivated and incubated in substrate to which is added alkaline phosphatase. Nucleoli, chromatin, and bands stain just as intensely as in nucleic acid-containing controls.

These experiments indicate, then, that it is by virtue of calcium phosphate formation in the medium rather than by phosphate liberation from the structural nucleic acids that the added enzyme produces nuclear staining.

Rat liver. The periductal capillaries and the bile canaliculi, although they show very high alkaline phosphatase activity in the standard Gomori procedure (Figs. 1 and 3), give no evidence of calcium phosphate adsorption (Figs. 2 and 5). There is no difference in the degree of staining of the hepatic cells of the periportal and central vein areas, such as occurs with the Gomori method (Fig. 1). Instead all cells are equally stained (Fig. 2). Nucleoli, chromatin, and nuclear membrane are black; the cytoplasm generally shows a diffuse light-gray color.

Guinea pig kidney. The brush borders, which are the most intensely stained structures with the Gomori technique (Fig. 7), show no evidence whatever of phosphate adsorption stain (Fig. 8). All nuclei stain darkly—in the distal tubules as well as in the proximal tubules, and in the outer layers as well as in the inner layers of perirenal fibroblasts.

Drosophila salivary glands. Where preservation of their alkaline phosphatase is unnecessary, the salivary glands are better dissected and squashed in 60% acetic acid rather than in saline. With saline the only chromosomes preserved are those remaining within nuclei, whereas with acetic acid large numbers of isolated chromosomes may be found on the slide. In such chromosomes it is apparent at once that the transverse bands show intense adsorption stain (Fig. 10). The absence of stain from the inter-band regions is especially striking in the chromosomes which chance to be pulled out to long threads. Nucleoli show little, if any, stain.

Nucleic acid and calcium phosphate adsorption. The high affinity of the nuclear structures for calcium phosphate is apparently not dependent upon the nucleic acids of these structures. The nucleoli of liver and kidney sections incubated in RNA-ase no longer stain with toluidine blue, yet they show undiminished staining with the standard Gomori procedure. The removal of Feulgen-positive materials by DNA-ase does not decrease the staining of either kidney and liver nuclei or *Drosophila* salivary chromosomes.[5] In-

[3] 0.1 mg RNA-ase (Worthington Biochemical Laboratory) per ml, in distilled water at room temperature for 3–6 hr.

[4] 0.1 mg DNA-ase (Worthington) per ml, in McIlvaine's buffer, pH 6.5, at room temperature for 5–7 hr in the presence of 0.01 *M* $MgCl_2$.

[5] The survival of typical alkaline phosphatase activity following treatment with RNA-ase and with DNA-ase (*cf.* 12) indicates that nucleic acid is not essential to the activity of the enzyme present in the sections and "squashes."

TABLE 1

EFFECT OF pH ON ADSORPTION BY NUCLEI OF INACTIVATED RAT LIVER SECTIONS*

Initial pH	3-hr incubation			19-hr incubation		
	pH at end of incubation	Stain intensity†		pH at end of incubation	Stain intensity†	
		Slide facing up	Slide facing down		Slide facing up	Slide facing down
10.01	9.73	0	1	9.36	1	5
9.71	9.43	1	3	9.19	2	6
9.60	9.31	1	3	9.14	2	6
9.49	9.25	2	3	9.08	3	7
9.41	9.21	2	4	8.98	5	8
9.31	9.10	3	4	8.90	5	8
9.12	8.97	3	4	8.80	8	10

* Calcium phosphate produced by addition of 90 μ *M*-units of alkaline phosphatase to 80 ml of incubation medium. Two pairs of slides in each beaker. Buffer: 0.05 *M* 2-amino-2-methyl-1,3-propanediol. pH measurements at 33°–36° C.

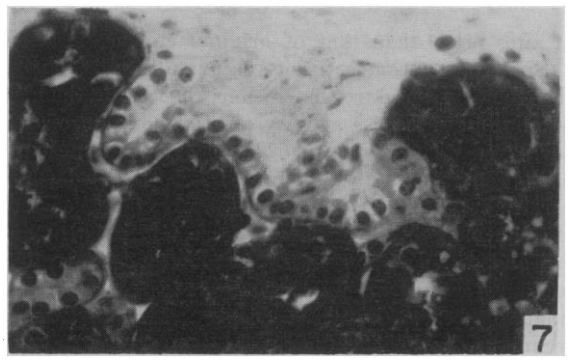
† Visual estimation: 1 (weakest positive reaction) to 10 (strongest reaction); 0 negative reaction.

activated sections and "squashes," treated with RNA-ase,[3] DNA-ase,[4] or 5% trichloracetic acid (90° C, 15 min) show the usual strong nuclear and chromosomal adsorption staining when incubated in the glycerophosphate substrate to which is added either alkaline phosphatase or hydrogen peroxide.

EFFECT OF pH ON NUCLEAR STAINING

These experiments are intended to test Gomori's recent conclusion (*13*) that adsorption artifacts do not occur at the alkaline pH of the Gomori-Takamatsu substrate medium. In the adsorption experiments above, the medium drops from an initial pH of 9.4 to a final pH of 9.0 when rat liver supernatant fluid is used (incubation time: 16 hr), to 8.6 with H_2O_2 (incubation time: 16 hr), and to 8.4 when 900 μ *M*-units (*14*) of alkaline phosphatase are used (incubation time: 30 min). It is, however, possible to maintain the pH of the medium more effectively with 0.05 *M* 2-amino-2-methyl-1,3-propanediol as buffer (*15*). Varying the final pH from 9.4 to 8.3 demonstrates a decided pH effect: adsorption stain increases in intensity with decreasing pH. Yet it is clear from experiments like the one summarized in Table 1 that strong nuclear adsorption occurs even with a final pH of 9.4. This is particularly true if the section is facing down during incubation (at an angle of about 45° between slide and beaker bottom) so that little calcium phosphate precipitate settles upon it.

In the standard Gomori procedure, with barbiturate as buffer and an initial pH of 9.4, the medium drops to pH 8.9–9.1 after 15–18 hr of incubation of 1–5 slides in a Coplin jar. However, by using propanediol buffer it is possible to test the effect on nuclear staining of a high pH through the entire incubation period. Above a final pH of 9.9 nuclear staining is absent from both kidney and liver sections. But at such high



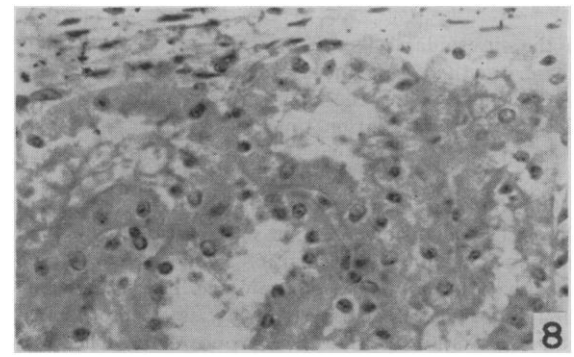


FIG. 7. Guinea pig kidney (× 400). Standard Gomori procedure. Incubation time: 17 hr.
FIG. 8. Guinea pig kidney (× 400). Inactivated and incubated for 16 hr, in 40 ml substrate medium to which was added 0.4 ml 3% H_2O_2.

pH's the alkaline phosphatase activities of the brush borders of the kidney and the periductal capillaries of the liver are also greatly diminished. Thus, nuclear staining disappears only when all enzyme activity in the section is greatly reduced.

Enzyme Adsorption

Repeated but unsuccessful attempts were made to demonstrate adsorption of alkaline phosphatase itself by inactivated sections. Wide variations were made in the concentration of alkaline phosphatase to which sections were exposed (up to 840 μ *M*-units/ml), in the time of exposure, and in the washing procedure following exposure. With the highest concentration of enzyme a diffuse black film is often present on the slide, indicating incomplete washing of enzyme from the slide. In these cases the nuclei show the typical "adsorption" stain. But it is clearly impossible in these cases to exclude calcium phosphate adsorption. When the enzyme has been washed off the slide sufficiently so that no sign of gross phosphate formation is evident, no staining of the nuclei occurs.

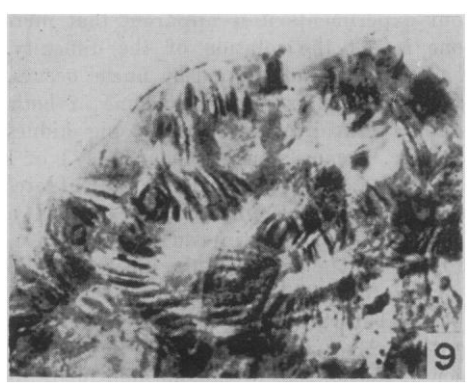
Azo Dye Method for Demonstrating Alkaline Phosphatase

The azo dye which forms in the method of Menten *et al.* (*16*) is more insoluble than the calcium phosphate of the Gomori procedure (*17*). Does it offer the solution to adsorption difficulties?

Inactivated kidney and liver sections show no adsorption staining whatever when incubated in the substrate medium of Mannheimer and Seligman (*17*) to which is added alkaline phosphatase (900 μ *M*-units to 40 ml medium). The added enzyme produces enough red dye to stain the glass slide, yet there is no reddening of nuclei or of other structures, even when sections are kept in the solution for many hours.

When kidney sections (not inactivated) are incubated in the Mannheimer-Seligman medium at 10°–13° C, a distinct pink color is produced in the brush borders after 1 min. Longer incubation intensifies the stain and produces what appears to be a spread of red color to the cytoplasmic granules in the immediate vicinity of the stain (*cf.* *18*). It also produces a nonspecific yellowing of the cells, particularly in the cytoplasm. But no appreciable pink color appears in the nuclei, even if the slides are transferred to fresh substrate 3 times at ½-hr intervals (total incubation time: 2 hr).

Incubation of liver sections for 20 min produces a positive reaction in the periductal capillaries and



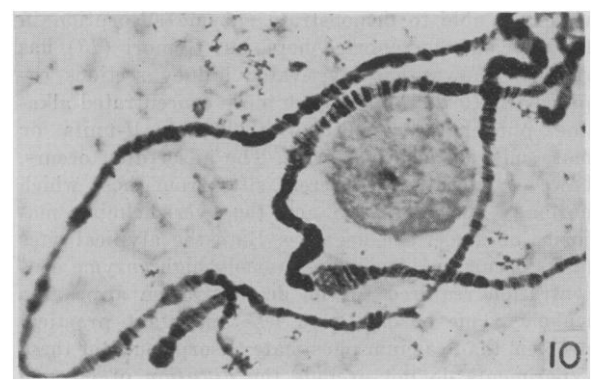


FIG. 9. Portion of flattened nucleus of salivary gland of *Drosophila pseudoobscura* (× 765). Standard Gomori procedure. Incubation time: 17 hr.
FIG. 10. Isolated chromosomes and nucleolus (× 765). Inactivated by 60% acetic acid and incubated for 23 hr in 40 ml substrate medium to which was added 0.4 ml 3% H_2O_2.

in the sinusoidal cells which stain intensely with the Gomori method. But nuclei give a completely negative reaction even when the substrate is removed 3 times and the total incubation time is 2 hr.

Discussion of Findings

Our findings make it quite clear that, contrary to Danielli's conclusion (*10*), the cytological sites of high calcium phosphate adsorption and high alkaline phosphatase activity do not coincide. Rat liver nuclei, though low in enzyme activity by direct biochemical assay, strongly adsorb calcium phosphate. The periductal capillaries of rat liver and the brush borders of guinea pig kidney are high in activity, as judged by staining with the Mannheimer-Seligman procedure or with short incubation periods in the Gomori technique; yet they fail to adsorb precipitated calcium phosphate under our conditions.

Although more pronounced at a lower pH, nuclear adsorption is still clearly demonstrable in media in which the pH never falls below 9.7. Under different experimental conditions, Gomori (*13*) has been unable to detect such adsorption above pH 8.5. It is difficult to see why one set of conditions is closer than the other to that which obtains in the customary Gomori technique. As we employ it, the pH of the incubation medium, beginning at 9.4, is about 9.0 after 16–19 hr of incubation. Yet our slides show apparent diffusion and adsorption by nuclei (See descriptions above and Figs. 6 and 7; also Fig. 4 in Robertson *et al.* [*7*]).

Noted earlier by other workers (*19, 20*), this diffusion phenomenon has been experimentally established in brilliant fashion by Martin and Jacoby (*21*). They also cite many instances in the literature in which nuclear staining is of dubious significance because it occurs adjacent to areas of high phosphatase activity.

It is not established whether it is calcium phosphate or enzyme or both which diffuse from regions of high alkaline phosphatase activity, to be adsorbed by nuclei. The literature has had little more than suggestions of diffusion and adsorption of enzyme (*2, 19, 21*). We were not able to demonstrate enzyme adsorption in the experiments reported here, but Gomori (*13*) has recently shown that inactivated kidney sections exposed for 10–20 min to much more concentrated alkaline phosphatase solutions (2,000 μ *M*-units or more/ml) do adsorb enzyme. The adsorption occurs, however, in entirely different sites from those which normally show activity, and the overwhelming majority of nuclei are negative. Both the atypical sites of its occurrence and the extremely high enzyme concentration required for its demonstration appear to make enzyme adsorption a less important practical problem than calcium phosphate adsorption. But these experiments do not exclude the *diffusion* of enzyme during the usual Gomori procedure. Were this to occur while the slides were in the substrate medium, calcium phosphate would be released in the medium at sites other than that of original enzyme location.

Since adsorption artifacts occur so readily during the Gomori procedure, conclusions concerning the high alkaline phosphatase activity of nuclei and chromatin compared with cytoplasm (*22, 23*) or of chromosomal bands compared with inter-band region (*6, 8*) demand careful scrutiny. As long as it is not established that the enzyme content of the cytoplasm in a particular cell and of adjacent structures is low, it is hazardous to conclude that the nucleus is rich in alkaline phosphatase because it stains deeply with the Gomori technique. As long as both cytoplasm and extra-band areas of the nucleus are not excluded as loci of appreciable alkaline phosphatase activity, it is equally hazardous to conclude that chromosomal bands are rich in enzyme because they stain intensely.

Also requiring closer scrutiny are the reports differentiating nuclear from cytoplasmic phosphatases (*19, 24, 25*) and the conclusions concerning the role of alkaline phosphatase in nucleic acid metabolism (*23, 26*) and gene action (*8*).

If, as our observations suggest, the azo dye method is free of adsorption difficulties, then a search for more stable diazonium compounds may prove rewarding. At present, incubation is limited to low temperatures (near 10° C) and short periods. Thus the failure of nuclei to stain may mean a low level of enzyme rather than its absence. The nuclear fraction obtained from rat liver by centrifugation has about 15% of the total alkaline phosphatase activity. Possibly half of this activity is due to whole cell and mitochondrial contamination of the fraction, leaving from 5–10% in the nuclei themselves. Is there any residual activity left in these nuclei at the end of the histological procedures or is the enzyme completely gone? If longer incubation periods and higher temperatures become possible with the azo dye technique, the answer may be forthcoming.

The Gomori-Takamatsu method cannot provide the answer to such precise localization problems unless the adsorption of calcium phosphate at sites other than those of enzyme location can be eliminated. From our experiments it is apparent that increased pH alone is not the solution of the difficulty, for even at pH 9.7 such adsorption by nuclei occurs. Unfortunately, above this pH the enzyme of both sections studied (rat liver and guinea pig kidney) is quite inactive. Gomori's suggestion (*13, 27*) of keeping the slides face downward only accentuates the calcium phosphate adsorption by inactivated nuclei under our conditions. Martin and Jacoby's proposals (*21*) of omitting Mg-ions from the incubation mixture and of drying the celloidin film over the sections in air for 3–5 min before hardening in alcohol are worth investigating. Most fruitful may be a study of the fundamental mechanisms of calcium phosphate formation, precipitation, and diffusion in the incubation medium.

It is possible that freezing or freeze-drying methods

may in some instances overcome the difficulties encountered in the usual histochemical procedures. By substantially decreasing the loss of enzyme, they may greatly shorten the time required in the incubation medium to bring out the activity of a given structure. The advantage of this in the azo dye method is apparent since the life of the diazonium compound is limited and the enzyme operates at suboptimal temperatures. That it may have advantages in the Gomori method, too, is suggested by recent work of Meath and Pope (*28*), who report that the most intense acid phosphatase staining occurs in the nuclei when the sections are of the customary fixed tissue, but in the cytoplasm when of unfixed frozen tissue.

It is too early to predict the extent to which omission of fixation and embedding can overcome the relative insensitivity of the azo dye method or the diffusion and adsorption difficulties complicating the Gomori method. These two methods at present are of little value for the localization of alkaline phosphatase on the intracellular level, except for structures with extremely high activity such as the brush borders of the kidney. For the moment it would appear that the actual isolation of intracellular constituents, although itself not free of pitfalls, offers the only means of localizing the enzyme inside the cell. The histochemical methods can, however, be used to advantage in indicating some pitfalls in the isolation method.

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Average Body Temperature in Mice[1]

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Estimates of average body temperature have been made for human beings (*1*) from skin and rectal temperatures. Few attempts appear to have been made to measure or estimate the average temperature of small mammals or birds, although determinations have been made of rectal, skin (*2*), esophageal (*3*), and subcutaneous temperatures (*4, 5*). In the present study a method was developed to investigate interrelations between the average body temperature of mice and environmental conditions, particularly during exposure to low temperatures.

The method[2] adopted was to place a mouse immediately after death in a Dewar flask calorimeter (218-ml) containing 100.0 g of water at a known temperature, record the temperature change, and calculate the average temperature (method of mixtures) after applying the thermometer and "radiation" corrections. The temperature change was determined by thermometers divided into intervals of 0.1° C, but estimations to 0.01° C were made with the help of a lens. Each thermometer was fitted with a No. 11 rubber stopper that closed the Dewar flask, and the lower end of the thermometer bulb rested about 5 mm from the bottom of the flask. The best results were obtained by carrying out the tests in a constant-temperature room at approximately 20° C. The initial water temperature in the calorimeter was 20.5° to 21° C, and the final temperature 22° to 23° C for normal mice, or 19° to 20° C for chilled mice (body temperature lowered). "Radiation" corrections were made from the rate of change in final temperature by the Regnault-Pfaundler or the Dickinson methods (*6*). During the tests, stirring of the water in the calorimeter was accomplished by means of a mechanical shaker. It took about 30 min to reach the final temperature after introducing the mouse. After mice were killed and equilibrated to a constant temperature in an air bath, the average body temperature did not differ significantly from the temperature of the bath when 0.83 was used as the specific heat of the mice. The standard deviation of determinations on 15 mice so tested was ± 0.25° C.

Tests to determine whether post-mortem heat production contributes appreciably to the results furnished evidence that heat production after death is small or negligible compared to the heat present in the body. The evidence is: first, the consistency of the average body temperatures, which generally fell between skin and rectal temperatures; second, killing mice by KCN injections gave similar results to killing by percussion; third, thermal equilibrium with the environment is established rapidly (1 to 2 hr) after

[1] Preliminary note.
[2] A more detailed description is in preparation.