A METHOD FOR PHOTOELECTRIC COMPARISON OF RELATIVE INTENSITIES OF THE HISTOCHEMICAL REACTION OF PHOSPHOMONOESTERASES, PARTICULARLY IN THE ADENOHYPOPHYSIS OF THE GUINEA-PIG

By

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With the aim of advancing cytochemical investigations on the distribution of phosphomonoesterases in the anterior lobe of the hypophysis of the guinea-pigs (Abolinš, 1948 b; Abolinš & Abolinš, 1949), attempt was made to introduce an objective method for the comparison of relative amounts of sulfide precipitates in sections instead of the usual visual estimation. The photoelectric measurement of the light loss in preparations was chosen because of the high order of the reproducibility of singular observations attainable in this way. The author is aware of the limitations inherent the method to be described and consider it as a first empirical approach towards an established microphotometric method.

Stowell (1942) was one of the first to describe a simple photoelectric microphotometer which seemed to be suitable for the measurements of the amounts of stain (Feulgen) or

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pigment in tissue sections. He used, for this purpose, a RCA 929 vacuum phototube connected with an amplifier tube, a potentiometer, and Leeds and Northrup galvanometer type R. A movable mirror mounted together with the phototube in a light-tight box, allowed the visual control of the field to be measured and the passage of light from the microscope to the photocell. The mean percentage-absorption of stained preparation was corrected by blank readings for non-specific loss of light of as well as by readings in unstained sections for specific tissue absorption.

Authors who subsequently made use of visual-range microphotometry in cytological investigations mostly employed the Electronic Photometer Mod. 512, manufactured by the Photovolt Corporation in New York. For the work in the visible part of the spectrum the search unit of this apparatus is also provided with the above mentioned phototube RCA 929, whilst another essential part of the equipment is the particularly high-gain DC amplifier. Pollister & Ris (1947) used the Electronic photometer to make the measurements both in the visible region (of Feulgen’s reaction, Millon’s reaction as well as for staining with pyronin and methyl green) and in the invisible region (for purine and pyrimidine determinations). An additional direct-current multiplier with a high vacuum photo-multiplier tube RCA No. IP28 was supplied by the Photovolt Corporation to be employed in the ultraviolet range. Dempsey, Bunting, Singer & Wislocki (1947) applied the Electronic photometer to evaluate the dye-binding capacity of mammalian mucopolysaccharides. While Stowell placed the sensitive surface of the phototube rather near the eyepiece, Pollister & Ris, and Dempsey et al. adapted the photometer to the microscope by picking up the area to be measured from a projected eyepiece-image at a level approximately 30 cm. above the eyepiece. A comparison of the results obtained by photometrical and chemical methods proved that the error inherent in the first was less than 10 per cent. Further must be referred to Di Stefano (1948) who used a photometric equipment in the estimation of the intensity of the Feulgen nucleal reaction.
Pollister & Ris (1947), Di Stefano (1948) and Stowell & Albers (1943) also discussed principles of absorption calculations and corrections in photometry of stained microscopic sections.

A detailed theoretical foundation of the microspectrographic analysis was developed by Caspersson (1936, 1940, 1950, 1950 a, 1951). The basic theoretical conditions for the microphotometric work have been further examined particularly by Engström (1946, 1947), Thorell (1947), Commoner (1949), Danielli (1949), Ris & Mirsky (1949), Pollister & Swift (1950), by the members of a General Discussion of the Faraday Society with the chairman J. T. Randall (1950), and recently by Glick, Engström & Malmström (1951). It has been proved by the investigations mentioned which a great number of precautions must be observed to avoid a faulty interpretation of the measurements of light loss, especially in highly inhomogenous stained preparations.

MATERIAL AND METHODS

1. Photometric Equipment.

The equipment used by the author of this paper corresponds in general to the construction adapted by Pollister & Ris (1947), and Dempsey et al. (1947). Some new arrangements have been incorporated to secure greater precision and efficiency. Fig. 1 shows the main features of the construction.

a) A research microscope with a cross-board is fitted with a rotating prism, which allows the image-beam to be deflected from the specimen alternatively to a binocular body-tube for subjective examination and to the monocular one for photometric and photographic recording. The light source, a Mazda tungsten ribbon 6 v. 108 W. lamp (not a common tungsten filament lamp), in a lamp-house with a condenser corrected for critical illumination and a pyrex water-cell, is connected to A. C. by a three-step transformer and a voltage stabilizer of the type GM 444 Svenska A. B. Philips.
b) The monocular recording body-tube is equipped with the Zeiss photomicrographic camera »Miflex« for negatives of the size 6.5 × 9 cm. The telescopic eyepiece allows for an efficient focusing of the image. Simultaneously with the insertion of the automatic exposure-shutter, the focussed image is projected on to the plane of the photographic plate or on to the plane of the recorder to be described below. When this arrangement was constructed and tested, we learned that Holter & Løwtrup (1949) successfully used the Photovolt Electronic photometer in combination with a similar Leica photomicrographic adapter in microchemical proteinase determinations.

c) To carry out photometric measurements, instead of photomicrographs, a simple photometric recorder was constructed by transforming a usual plate-holder. Two modifications of such a recorder were used. (1) The slide of the plate-holder having been removed, a clear-glass plate is inserted at the place of the photographic plate. The back- (upper) wall of the plate-holder is altered to a ground-glass pusher. (2) A metallic screen with a graduated iris diaphragm in the center is inserted into the plate-holder instead of the photographic plate. As in (1), the back-wall is altered to a ground-glass pusher. In (1), the image of the specimen is picked up by the phototube from the clear-glass; in (2), it is projected through the opening of the diaphragm direct on to the window of the phototube. The distance between the eyepiece and the image focussing-plane of the »Miflex« is equal to 15 cm. (multiplication index 0.5) and can be lengthened by additional camera adapter pieces.

d) A movable heavy pillar with a horizontal arm bears the photometer search unit with the phototube. The inner bar of the pillar can be lifted and lowered by the screw with a winding alley and brought to a stop by another screw. In the arm on the top of the bar there is inserted the shaft of the search-unit holder. It can be moved forwards and backwards, turned around the axis and stopped by a screw. The search-unit holder itself is a cross-board on which the mounted search-unit can be shifted by fine movements of the adjusting screws.
Fig. 1.

Diagram of the photometric equipment. A. C. S. — voltage stabilizer, l. s. — light source, m. — mirror, m. d. — condenser diaphragm, C. — condenser, o. s. — cross board with the specimen, o. — objective, s. e. — light-beam from the rotating prism of the microscope to the body tube for subjective observation, e. i. p. — eyepiece image plane, e. d. — eyepiece diaphragm, t. e. — light-beam from the rotating prism of the »Miflex«-adapter to the telescopic eyepiece, c. — camera, m. f. — image projection on the clear-glass screen of the photometric recorder, u. sh. — ground glass pusher, p. p. sh. — metallic screen of the second type photometric recorder with the diaphragm — d., c. m. — camera mirror, c. w. — camera window, s. u. — search unit, s. u. d. — unit diaphragm, e. p. — extension piece, s. u. c. b. — search unit cross board, s. u. s. — horizontal arm of the pillar, A. and G. — Electronic photometer device.
The construction allows the precise centering and adjustment of the window of the phototube upon the focussed image of the specimen on the ground-glass of the recorder only by eye-control from above. To facilitate the centering and the elimination of side-light, the extension pieces (with or without ground-glass in openings) can be placed on the phototube window. Their openings must closely circumscribe the measured areas. The extension pieces can be connected to the phototube window together with an iris diaphragm. This is an advantage, especially when the clear-glass recorder without the diaphragm is employed (though the diaphragm supplied for this purpose by the Photovolt Corporation is inconvenient to use because of its uneven action and lack of graduation).

After adjusting the phototube on the image of the recorder, the ground-glass pusher is drawn out and the search-unit lowered still more until contact is reached with the plane of the clear-glass, or the opening of the diaphragm of the recorder. Note that the ground-glass is used only to visualize the projected image in the recorder, thus facilitating the adjustment of the phototube. A light-tight cloth jacket protects the contacts between the extension piece and the recorder, as well as those between the camera and the microscope from disturbing side-light during the measurements. It is recommended to carry out the readings on the Electronic-photometer indicating instrument with the aid of a magnifier, since the scale of the instrument is too small. Once adjusted, the search-unit can be kept in this position and only raised when the photometric recorder must be changed for the usual plate-holder to take a photograph of the specimen measured. Thus, the arrangement allows the carrying out of (a) subjective examination as well as (b) a photometric and (c) a photomicrographic recording of a specimen alternatively.

The photometric equipment outlined above was constructed in 1948; it has continuously been used since that time, without any trouble. In 1949 Pollister & Moses (1949) described an apparatus for photometric and photomicrographic work which may be regarded as a further development of the pre-
viously mentioned apparatus by Pollister & Ris (1947). The very exactly constructed image-centering and -focussing device of this apparatus allows the use of the search-unit without extension pieces. Moreover, there is no need to raise and lower the search-unit when photomicrographic and photometric recordings are to be made alternatively. It is obvious that the apparatus is of great advantage, especially when measuring small areas, and in the ultraviolet range. However, a comparison of the details of the apparatus with those of ours make us believe that the final efficiency of both might be of the same range. In the present outfit also the apparatus by Pollister & Moses hardly can replace the apparatus for visual and ultraviolet microspectrography, developed by Caspersson (1936) or that for ultramicrospectrography by Caspersson, Jacobson & Lomakka (1951) when very small, optically really homogenous, individual areas of cell structures have to be measured.

The opportunity to use the microscope of our equipment simultaneously for routine microscopy, drawing counting etc., without disarranging the centering of the phototube upon the image of the visual field on the recorder, has proved to be a gain.

2. Histochemical Technique.

Hypophyses of guinea-pigs were fixed immediately after their removal, for the examination of alkaline phosphatase in chilled acetone, or 96 per cent alcohol, and for that of acid phosphatases in chilled acetone. Gomori's (1941, 1946) method with some modifications by Kabat & Furth (1941), Wolf, Kabat & Newman (1943), Danielli (1946) and Abolins & Abolins (1949) was used to visualize the activity of phosphonoesterases cytochemically, in the anterior lobe of the hypophysis. Glands embedded in paraffin were cut frontally at 4 µ; testing linearity — at 4—12 µ. Sections, attached to slides with destilled water, were incubated for alkaline phosphatase at pH 9.7, for acid phosphatases at pH 6.6 and 5.5. The incubation substrate was β- and α + β-glycerophosphate.
The incubation times were 30 min., 1 hr., 24 hrs., 48 hrs., 72 hrs. Preparations were mounted in neutral Canada balsam. Gomori (1948, 1949, 1949a, 1950), Lison (1948), Manheimer & Seligman (1948), Seligman & Manheimer (1949), Jacoby & Martin (1949), Martin & Jacoby (1949), Ruyter & Neumann (1949), and Novikoff (1951) have recently critically examined the cytochemical phosphatase visualization technique. Also the author of this paper has worked with regard to the standardization of the technique. A more detailed description of our modifications, diffusion controls etc. will appear in another paper.

3. Choice of Sections for Measurements.

As the aim of our photometric investigations for the present has been (a) a record of the relative enzymatic activity of the adenohypophysis and (b) a comparison of this activity in various glands, it was very important to choose for measurements such sections as were representative of the topography of all cell types in the gland. The peculiarity of the topography of various cell types of the anterior lobe of the hypophysis of the guinea-pig has been described particularly by Kirkman (1937), Krogis (1939), Abolins-Krogis (1946), Giroud & Desclaux (1947) and Abolins (1948). The majority of acidophile cells occupy the lateral parts of the adenohypophysis with a gradient rising towards the caudal end. The majority of basophile cells are concentrated peripherally and centro-ventrally with a gradient rising towards the stalk of the hypophysis. The chromophobe cells are distributed rather irregularly between both the other types over the whole pars anterior, though in greater number in the area of the basophils. To ensure that the measurements should be comparable, the paraffin ribbons of all hypophysis investigated have been divided into four segments. The border-zone between every two segments gave, in all glands, the corresponding typical cranial medial, and caudal sections. For details of the determination of border-zones, see the original paper (Abolins, 1948). Fig. 2 shows the approximate positions of these borders. From every hypophysis a
number of sections from each zone were divided into pieces containing two sections. A piece, or two, from each zone was mounted on a number of slides. Slides were incubated for various kinds of phosphatases, at various pH, with regard to different reaction times, in order to determine the effect of the thickness of the sections etc. Knowing the sequence of the sections on the slides, it was possible, by this technique, to compare the enzymatic reactions a) in adjacent sections of the same hypophysis under varied conditions and b) in corresponding zonal sections of various hypophyses. Moreover, it proved to be possible to record, by photometric measurements, all three cell types of various sections in their true relations. For this purpose the measurements were carried out in one typical section of each of the three zones of every pituitary. In every section measurements were made of three definite files of square visual fields by using an ocular diaphragm of 16 sq. mm. and the adjusted cross-board of the mechanical stage. The optical system employed was a stage condenser of n. ap. 1.2, an objective of n. ap. 0.85 (45 ×) and an eyepiece of 5 ×. Fig. 3 shows the position of measured visual fields in a topographical standard scheme, and a corresponding photomicrograph from a cranial section of the pituitary. As the cell topo-
graphy in both lateral parts of the pituitary is not entirely adequate, it is recommended to obtain measurements on the whole surface of a section, i.e., in five files instead of three. However, the difference resulting from measuring three and five files is for the most part insignificant.

By the method described, 15—20 visual fields (depending on the size of the pituitary) were measured in each section. A mean of three, twice corrected (see below) absorption values of the same field represented this field. A mean of the absorption values from all fields measured in the section was considered as the absorption value of the section. A mean of the absorption values of all three zonal sections was regarded as yielding the absorption value of the whole pituitary, i.e., its relative phosphomonoesterase activity as measured by the light loss in the absorbing precipitate formed in sections during enzymatic reaction with a definite substrate, at definite pH, temperature, and incubation time. Such absorption values were further used for the construction of curves. It may be mentioned that, in the same pituitary, the absorption value of the medial zonal section alone is often almost equal to the mean absorption value of all three zonal sections. This seems to be a result of the previously mentioned reciprocity of cranio-caudal gradients in the distribution of acidophile and basophile cells in the anterior pituitary of the guinea-pig. Whenever possible, however, the mean of all three sections must be taken as representing the absorption value of the whole hypophysis.

**DISCUSSION ON THE PHOTOMETRIC METHOD USED**

1. **Corrections for Errors.**

The general conditions for absorption photometry (Harrison, Lord & Loofbourow, 1948; Brode, 1945; Drabkin, 1944, 1950; Reich, 1941; Sawyer, 1944; Sawyer & Vincent, 1941; Vincent & Sawyer, 1942) have been analyzed by a number of authors, mentioned even in the introduction, particularly in relation to the microphotometry of biological specimens.
The technique described in this paper satisfies the demands at the following rate:

a) The phototube RAC 929, having its main sensitivity in the blue and near ultraviolet, compensates the relatively low radiation intensity of the tungsten lamp in the blue-violet. The influence of infra-red radiation is excluded by the complete insensitivity of the phototube in this region. The influence of temperature on the specimen is largely avoided by the heat-absorbing water-cell (especially if filled with CuSO₄-solution filter) placed in the path of the light-beam.

b) Stray light from the room is eliminated by screening the apparatus by a curtain, or placing it in a wide case open in the front. This precaution also reduces the accumulation of dust in the light path, i.e. reduces the scattering of light by the optical system of the apparatus.

c) Symmetrical orientation of the light-beam around the optical axis of the apparatus, as well as continuous uniformity of the illumination of the visual field by parallel rays, is greatly facilitated by the use of (a) a tungsten ribbon lamp having a very even luminous surface, (b) a lamp condenser corrected for critical illumination, (c) a voltage stabilizer, and (d) the high sensitivity of the phototube. This installation makes it possible to obtain the measurements with a relative narrow stage-condenser diaphragm without difficulty, and thus keeps the energy falling on the specimen relatively low. An extremely narrow stage-condenser diaphragm, however, gives disturbing diffraction phenomena (Caspersson, 1950). To avoid this, use has been made of a stage-condenser diaphragm having an opening between the medium and minimum width. To make the measurements, in spite of this, within the highest sensitivity range of the Electronic photometer (ratio 15:1), two light reducing filters, a day-light glass-filter (Corning, Daylite No. 5900), and a Wratten gelatin film Neutral density filter, dens. 0.7, transm. 20 p. c. (accuracy 5%), were utilized together with the water-cell. Since they were used both in test and blank readings, their calibration by the optical system employed was, for the time being, omitted.
d) The optical isolation of the area to be measured can be secured in two different ways. If it is necessary to obtain comparative measurements of preparations at a constant image surface, an appropriate rectangular diaphragm can be placed on the image plane of the eyepiece. It is a method often used in cell-counting technique and applied in microphotometry even by Stowell (1942). A very important condition for the correct function of the vacuum tube is the maintenance of its sensitivity constancy by continued, uniform irradiation of as large a portion of the cathode-surface as possible. For this reason, the size of the rectangle projected on the focussing plane of the photometric recorder must be of a magnitude not exceeding the surface of the phototube window (in RCA 929 equal to 62.7 sq. mm.), and admitting some »diaphragmation« through the iris of the extension piece.

The isolation of smaller areas down to certain tissue structures is attained by the appropriate centering and »diaphragmation« of the projected image of the specimen in the focussing plane of the photometric recorder itself. If the clear-glass screen recorder is used, an appropriate mask can be placed on the screen to limit the centered structure. When the area of the limited image projection is very small and relatively obscure (as with high power magnifications), some difficulty can arise in centering the extension piece of the phototube on the area only by eye-control from above. It has been searched to avoid this difficulty by additional constructions. These constructions, however, need further testing.

For technique checking the uniformity of illumination and the accurate choice of the phototube diaphragm, a paper by Pollister & Moses (1949) may be consulted.

e) The influence of non-specific loss of light in preparations produced by the refraction and scattering of light in mounting media (slide, cover glass, Canada balsam) is abolished by readings in blank portions of the slide, adjacent to the mounted tissue section, as already practised by Stowell (1942). There is another source of error inherent in all microphotometric work with biological specimens. It is the »pseudo-
selective absorption caused by light refraction and scattering in »white cols«, i.e. in protein structures of fixed specimens. The problem has been analyzed in great detail by Caspersson (1932, 1933 a, b, 1936). We corrected each reading for this error, as usual, by reading in an unstained section close to the test section. It must be emphasized that the »unstained« section passed through all the chemical procedures of the »stained« one, only had none substrate to react with during the incubation. We must realize, however, that corrections for non-specific light loss as well as for specific (»pseudo-selective absorption«), are purely empirical procedures adopted from colorimetric practice. It might be of value to determine for each particular specimen, in a suitable way, the real magnitude of both errors mentioned above. Estimated empirically, as the difference between the readings in blank and unstained preparations, the error due to the pseudoselective absorption of the tissue itself was in the sections of the hypophysis equal to ca. 0.5—1.0 p. c. of the blank transmission. In practice the correction of readings (blank, unstained tissue), and the calculation of absorption (A), transmission (T) and extinction (E) values followed the formulae derived from the Lambert-Beer's law:

\[
A_{\text{test corr.}} = \frac{(T_{\text{blank}} - A_{\text{tissue}}) - T_{\text{test}}}{T_{\text{blank}}} \times 100; \\
T_{\text{test corr.}} = 100 - A_{\text{test corr.}}; \\
E_{\text{test corr.}} = \log \frac{100}{T_{\text{test corr.}}}. 
\]

The blank transmittance value of the slide was always adjusted as equal to 100 of the galvanometers scale.

f) The absorption measurements in cobalt and lead sulfide precipitates, formed by the reactions adjoining the enzymatic action of phosphomonoesterases, involve a further source of error, i.e. the refraction and scattering of light by these particles, deposited in tissue structures. It is not known how this
Fig. 3.

Above — photomicrography of a frontal half-section from the cranial zone of the pituitary of the guinea pig. Section has been incubated for alkaline phosphatase for 2 hrs. p. n. — pars nervosa surrounded by the anterior pituitary. Dark — predominantly acidophilic area, light — predominantly chromophobe-basophilic area. Below — diagram of the section. v. — ventral side, fine dots — acidophilic area, α — mean region of acidophilic degranulation, coarse dots — mean region of the pyknosis of acidophiles (Abolins, 1948 a). β — chromophobe-basophilic area. Rectangles show visual fields chosen for photometric measurements.

Error may interfere with the error due to the pseudoselective absorption of the tissue itself. Our measurements hitherto have not been corrected for the error due to the light scattering by sulfide particles. Consequently the figures of real absorption values in our preparations might be to some degree lower than those (twice corrected) obtained by direct readings.

While the error due to the pseudoselective absorption of the tissue itself is relatively insignificant in our preparations,
the error caused by the light scattering in precipitates might be of a much higher order. It might be also of different magnitude following different length of incubation, and at various pH. Both factors involve changes in the magnitude of solubility products of electrolytes taking part in reactions, i.e. in the size of the particles and in total amount of precipitates. In the continuation efforts must be made to verify the magnitude of the error due to the light scattering by sulfide particles. From the work with stained specimens, done hitherto, it is hardly possible to get some information about the order of light loss due to the physical interference of dye particles.

(g) The main source of errors in microphotometric measurements is the inhomogeneity of the optical field, characteristic of all biological specimens (Caspersson, 1936, 1950, 1951; Glick, Engström & Malmström, 1951). In our case the inhomogeneity, both in the plane perpendicular to the beam (variable distribution of the precipitate upon different cell types and structures), as well as along the axis of the beam (multilayer distribution of cells in section), is also produced by sulfide particles. The error by inhomogeneity cannot be avoided if measuring, as by our procedure, areas containing each varied number of cells. In order to secure the value of the method described here, it will be of particular significance to establish especially the magnitude of this error. Further, it is obvious, that for quantitative evaluation of absolute intensities of enzymatic reactions and their gradients in singular cell structures, only methods like that by Caspersson, Jacobson & Lomakka (1951) can secure fully reliable results.

RESULTS. VALIDITY AND LIMITATIONS OF THE METHOD

Despite of some theoretically presumable errors of not yet established magnitude, which are inherent in the photometric procedure described, and can not be eliminated by the precautions and corrections used, the empirically attained results might be of interest.
(a) Repeated measurements in adjacent sections of different thicknesses from pituitaries both in normal and experimental conditions reveal that the extinction values within definite limits follow Lambert-Beer's law. Fig. 4 reproduces curves obtained in two very typical individual cases after incubating sections for alkaline phosphatase (pH 9.7) for various incubation times. For each incubation period sections have been used 1) from medial, and 2) from cranial or caudal zones of the hypophysis. For incubation times of 1 hr. and 2 hrs., the values approximate a straight line, as is to be ex-

**Fig. 4.**

Typical distribution of extinction values of the reaction of alkaline phosphatase in sections of the anterior pituitary of the guinea pigs, cut at 4—12 µ and incubated for times of 0.5, 1, 2 and 24 hrs. Hollow marks denote values from medial sections, black ones -- from cranial or caudal sections.
pected on the basis of Lambert-Beer's law. Shorter incubation results in lower values, especially for thinner sections, as it could be expected. A similar, yet reverse and more marked discrepancy with regard to the Lambert's law, is shown with incubations of long duration, e. g. 24 hrs. The causes of these discrepancies are not yet fully clear to us. The very long incubation time may induce reactions other than enzymatic involved in the true tricalcium phosphate deposition. Gomori (1950) showed that long incubation time can lead to supersaturation of the incubation medium with products of enzymatic and bacterial hydrolysis, i.e. to calcium deposite artifacts. Moreover, processes of diffusion can also alter the normal localization of the cytochemical reaction. Yet, the surprisingly good results of the measurements on preparations incubated for 1 hr. - 2 hrs. periods seem to show that the production of precipitates in sections must proceed with considerable regularity, and that the grain of the particles must be fairly uniform. Mean extinction values would give still greater agreement with the Lambert's law. However, considering the well known variability of the functional state in various hypophyses and the changing percentual content of different types of cells at various levels of the same hypophysis, it is not advisable to use mean values in this case.

In Fig. 5 extinction values obtained with regard to the acid phosphatase of pH 5.5 are plotted. An incubation for 72 hrs. shows almost satisfactory agreement with the theory. Though not shown in the figure, the incubation for 48 hrs. also gives accordant values. Incubation for a shorter time, as, for instance, for 24 hrs. and 12 hrs., gives (if there is reaction at all) mainly linearly distributed values, but not in accordance with the theory. It must be emphasized that the particles of lead sulfide deposited in tissues in consequence of acid phosphatase action are much coarser than those in the case of alkaline phosphatase. Thus, the error resulting from the scattering of light in the precipitate might be greater. Moreover, the histochemical reaction of the acid phosphatase has always been regarded, to some extent, as unreliable. This unreliability may
Typical distribution of extinction values of the reaction of acid phosphatase (pH 5.5) in sections of the anterior pituitary of the guinea pig, cut at 4—12 µ and incubated for periods of 12, 24 and 72 hrs. Hollow marks denote medial sections, black ones — cranial sections.

be caused, at least to some extent, by the fact that at lower pH values there is more chance for disturbances of the relation between phosphate (sulfide) ion concentration and the solubility product which involve the formation of precipitates. Further stabilization of the cytochemical reaction, especially for acid phosphatases, is still needed. Nevertheless, repeated controls have shown that, though not with the same degree of certainty, the photometric comparison of relative amounts of precipitates in sections, can within definite limits also be applied to the acid phosphatase. The validity of Lambert's law for sections thinner than 4 µ and thicker than 12 µ has not
been proved, since all our investigations on phosphatases in the hypophysis have been carried out with sections of 4 µ.

In the case of short-time incubations, both for alkaline phosphatase (0.5 hr.) and for acid phosphatase (12—24 hrs.), the real extinction values would form a curve of very typical bowed shape. A similar, bowed, curve, but of opposite shape, would form extinction values for alkaline phosphatase incubated for very long period (24 hrs.). Whether the shape of such curves is determined mainly by the interaction of chemical factors influencing the formation of precipitates, or by physical factors, such as variant degrees of inhomogeneity, changed conditions for light scattering etc., or by both, we can not decide at present. Kortüm & Seiler (1939) showed how different physical and chemical factors even in solutions of electrolytes can cause deviations from the rectilinear shape of the curve for Lambert-Beer's law. According to Caspersson, and Glick, Engström & Malmström (1951) the deviated shape of the curves discussed above might be determined mainly by the product of sulfide concentration (amount) and the degree of the inhomogeneity of the distribution of precipitates in sections.

(b) The validity of the microphotometric method for the estimation of the activity of phosphomonoesterases, naturally depends upon the accuracy of the histochemical method used. Every progress in the latter will also raise the efficiency of photometric measurements performed on the precipitates. The photometric estimation of relative intensities of phosphatase activity in sections is based on the assumption that the amount of the precipitate, formed in the course of the histochemical reactions in a structure, is strictly proportional to the amount of the active enzyme in it. We have seen, that there are a number of factors which influence this proportionality. However, by observing some precautions, it has been possible to obtain phosphatase activity curves, which, notwithstanding the presumed variations in cell content, are surprisingly similar for hypophyses of different individuals of similar sex and age. Fig. 6 shows an example of such curves which will be dis-
cussed in detail in another paper to be published. It seems that the comparison of phosphatase activity curves from different individuals exhibit good control for the reliability of histochemical reactions performed. In this connection, it may be remembered that curves constructed in respect to the Lambert's law appear to reveal the optimum incubation time for alkaline and acid phosphatases, i.e., the incubation time at which the factors disturbing the normal formation of precipitates are in minimum (1—2 hrs. for alkaline phosphatase).

Moreover, measurements carried out on hypophyses of experimentally treated animals (paper in preparation by Anna Abolins) show that the activity curves of phosphatase from various animals under similar conditions also are in agreement, and therefore of great value in the analysis of secretory behaviour of the glands.

(c) The measurement of adjacent sections gives, in the case of alkaline phosphatase, as well as acid phosphatase, absorption values which do not differ by more than ±5.0 per cent; and varies most frequently by ±1.0—2.0 per cent. Corresponding to the volume of cells in the anterior lobe of the hypophysis, only a part of the cell stock is common to both sections and can potentially produce an equal reaction. Under such conditions the very slight difference in absorption values of adjacent sections can be considered an additional argument for the relative stability of the histochemical reaction and for the reliability of the comparative method used.

(d) Repeated readings on the same rectangular area of the section, if carried out within not too long a time-interval (risk of bleaching of the precipitate), show differences lower than ±0.1 per cent. By careful observation of the charge of batteries of the amplifier, it is possible continually to maintain this high degree of reproducibility of singular measurements. The acceptable degree of reliability of cytochemical reactions and light loss measurements allows to take into consideration a further development of this summary method to a method for the comparison of enzymatic reactions in singular cells of different type.
It is apparent from what has been discussed under (a), (b), (c) and (d), that the validity of the method described in this paper is given for certain ranges of pH and incubation times by the relatively high degree of the reproducibility of measured values both for singular measurements in the same and in adjacent sections, and for comparative measurements in corresponding zonal sections from various hypophyses. The limitations of the method are made by the present lack of sure knowledge about the range of errors involved especially by the inhomogene distribution of precipitates in cell structures to be measured. Theoretically, the degree of the accuracy of the method is at present unknown. In practice, in comparative measurements under controllable conditions, the interference of the error due to the inhomogeneity, however, can be greatly reduced. We have seen that measurements on sections of hypophyses of different normal individuals give very similar, reliable values if the fields to be measured (involving various degrees of inhomogeneity) in all the hypophyses are chosen as possible similarly and most closely to the true topography of different cell types.

There are some possibilities of further developing the efficiency of the photometric technique described in this paper. It would be important to reduce the measured area as much as possible.

It might be of advantage also to make the measurements by using a monochromatic light source instead of the tungsten lamp, since, à priori, a better agreement with the Lambert-Beer’s law might be expected (Kortüm & Seiler, 1939). To restrict the loss of light by refraction (Caspersson, 1932, 1936), the use of the longer wave length would, presumably, be most suitable.

During recent years methods have been published concerning direct micochemical phosphatase determination. Gottschalk (1948) described a modification of the standard method by King & Armstrong (1934) suitable for determining the activity of acid phosphatase in an isolated tissue section by extracting it in a frozen-dried state with 10 μl of water. Kroon,
Neuman & Veerkamp (1948) described another micro-method which offers the possibility of determining the activities of various phosphatases in 10 µl of tissue extract obtained by extracting entire organs or small parts of them. Doyle (1950, 1951) proposed two methods for phosphatase estimations in histological sections. Doyle's method of direct assay of phosphatase activity in sections is also a modified King-Armstrong's method. Using Doyle's indirect method, the lead sulfide, formed in sections by the enzymatic activity, is extracted, converted to methylene blue and measured photometrically. The three methods of direct phosphatase microestimation, mentioned, are valuable in attaining a summary record of the activity of enzymes in sections. Yet, with none of them can a comparison of the relative intensities of the reactions localized in different cellular structures of the same section be made. The indirect method of Doyle is of great interest to the cytologist, since it allows the investigation both of the localization of the reaction in the section, and the summary recording of the enzymatic activity. However, it must be emphasized that the method involves all the sources of errors inherent in the technique of the histochemical visualization of phosphatases. The really original, purely cytological, method of Gomori (1950) for the quantitative determination of histochemical reactions for enzymes depends on the use of standard slides for comparison of the reactions in test slides. These standard slides bear marks of known enzyme solutions, diluted in gelatin, and are incubated together with the test slides. The estimation of enzymatic activity by comparison of the intensities of reactions in standard and test slides by eye-control is, however, also under the best conditions limited by the Weber-Fechner's law. Attempts have been made to complete the photometric method described here by methods of microchemical phosphatase estimation in sections. Calibration of curves, obtained in photometric estimations, for definite phosphatase activity units might be the most convenient way to advance the accuracy of this method. Another way is given by the computation of the magnitude of above discussed errors for inhomogeneity
Enzymatic hydrolysis of β-glycerophosphate by the alkaline phosphatase in the anterior pituitary of the guinea pig. Values of light absorption by sulfide precipitates in sections are plotted in relation to the incubation times of 0.5—72 hrs. Broken lines — single curves from pituitaries of four normal male animals weighing about 350 gm., unbroken — curve of average values.

It is also obvious, that, with regard to the needs of cytological investigation, the photometric method can not be replaced by the direct microchemical enzyme determination alone.

With regard to the limitations discussed here, it might be finally stated that the method for photoelectric estimation of relative intensities of the activity of phosphatases described here, is for the time being an empirical method for the comparison of singular reaction rates, however, with a high degree of reproducibility and prospects of being advanced to
a quantitative method with established order of magnitude of errors. Developed to examine the cytochemical phosphomonoesterase reactions in the hypophysis, it can, probably, be applied to the estimation of these reactions in other organs, and, may be, also to the evaluation of other cytochemical reactions. Essentials to this, however, are a high degree of constancy, i.e., of reproducibility of the reaction itself, and a careful, appropriate choice of areas to be measured in sections, with regard to the topography of cells in the organ.

When this paper was written, we learned of a short communication by Neumann (1949) who also describes the application of the photometric method in the estimation of phosphatase activity in tissue sections, using for this purpose a photocell, a mirror galvanometer, and an incandescent lamp or a carbon-arc as light sources. It is difficult to consider the validity of this method, without knowing more details about the extent of the standardization of the histochemical reaction, the kind of tissues examined, and the specifications of the photocell, light sources etc. The writer of this paper is doubtful as to the limits of errors calculated by the author.

**SUMMARY**

A photoelectric equipment was used to measure the relative light loss caused by sulfide precipitates formed in sections of the anterior lobe of the hypophysis as a result of the enzymic action of alkaline and acid phosphatases. The equipment consists of a research microscope fitted out with a cross board and a tube with rotating prism, a »Miflex« camera, a specially constructed simple photometric recorder and an Electronic Photometer (Photovolt Corporation, type 512) with vacuum phototube and high-gain D. C. amplifier. The light source, a Mazda tungsten ribbon lamp, is connected to A. C. by a voltage stabilizer. The arrangement allows the performance of a subjective examination, drawing, a photometric, and a photomicrographic recording of specimens alternatively.

The cytochemical technique, used, was that of Gomori with
some modifications. A method was elaborated to choose the sections and visual fields in them for measurements, with regard to the topography of different types of secretory cells in the gland. Sources of errors, validity of the method and possibilities of further developing the efficiency of the method are discussed. The extinction values, especially those obtained in incubating for alkaline phosphatase during 1—2 hrs., apparently follow Lambert-Beer's law within definite limits. It was possible to maintain a high degree of reproducibility in singular observations (± 0.1 per cent) as well as in comparison of reactions in adjacent sections (± 1.0—2.0 per cent), and in various hypophyses. The range of the absolute accuracy of measurements, however, is not yet determined. The method can be applied, probably, to the comparison of relative intensities of the reactions of phosphatases also in other organs.

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