The cysticidal activity of compounds. The method mentioned above is used to test the sensitivity of the trophozoites of the amoeba and is not applicable for cysts of the amoeba. Cysts of the amoeba, however, are readily obtained, and in abundance, after 48 h incubation at 37°C.

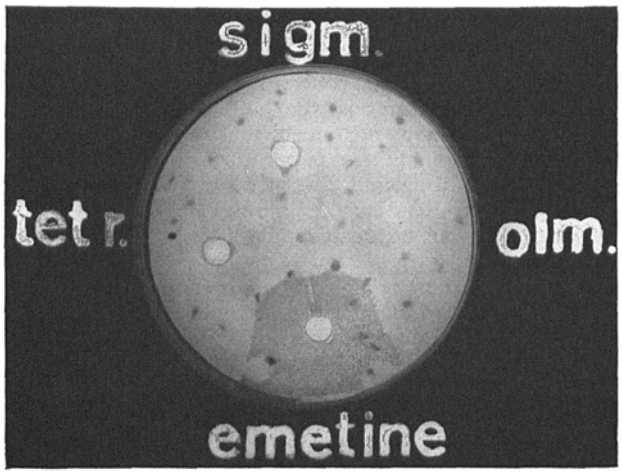

Fig. 3. Assay of amoebicides against the surface growth of the same amoeba in Figure 2. The amoeba was inoculated in many foci all over the plate marked by the opacities (due to bacterial growths). The plate was photographed by transmitted light. An 'inhibition zone' is noted within an area surrounding 'emetine'. The sectors of 'sigm' (sigmamycin), 'olm' (oleandomycin), 'tetr' (tetracycline) are devoid of any direct amoebicidal activity. The result was read here after 24 h incubation only, instead of 40 h as in Figure 2, because multifocal inoculation was used instead of the unifocal central inoculation used in Figure 2.

Saline is used to emulsify the cysts of the amoeba lying motionless on the surface of the agar with the aid of a platinum loop or a spreader as used in the preparation of bacterial vaccines. The suspension is then centrifuged at 1000 RPM for a few minutes and the supernatant is then discarded. Fresh saline is added to re-emulsify the cysts in the tube for recentrifugation. This is repeated 3–4 times or until the supernatant saline becomes perfectly clear. On a given cyst suspension (1,000,000 cysts/ml, for example) the cysticidal power of a test compound in a given concentration (1/1000, for example) after a known period of exposure and at a given temperature and the pH, can be determined. At the end of the exposure, the cysts are washed in saline and are tested for viability by staining and culture. Cysts that take the stain (eosin 1/1000) are considered non-viable. A loopful of the washed, exposed cysts is also inoculated on a fresh warm medium, prepared as usual, and watched for growth for 24–72 h before giving any report about the cysticidal power of the compound.

Growth stimulating substances. Whereas the spread of the amoeba towards a disc impregnated with an amoebicide is retarded, it was noted that the reverse is true, i.e. the spread is accelerated towards a growth stimulating substance.

Résumé. Une nouvelle méthode de culture d'amibes sur plaques d'agar (Figure 1) est avantageusement employée ici en vue de la détection rapide et facile de l'action antiamibienne directe des composés (Figures 2 et 3).

K. A. YOUSSEF

Ministry of Public Health, Cairo (Egypt), February 10, 1964.

Continuous Optical Determination of Alkaline Phosphatase[1]

The possibility of a direct and continuous optical measurement of an enzymic reaction rate by recording the formation or disappearance of a chromogen with a recording photometer generally offers advantages in accuracy, precision and rapidity and allows a better control of kinetic behaviour.

A special substrate, phosphorylmonosalicylate, was proposed for measurement of phosphatase activity with direct and continuous optical methods[2,3]; but, at alkaline pH, a suitable substrate for such methods is also *p*-nitrophenylphosphate, very commonly used for phosphatase activity assay[4–6].

In the present paper a continuous optical procedure for the determination of alkaline phosphatase with *p*-nitrophenylphosphate as substrate is described in detail; this procedure has been applied to research concerning the adaptation of this enzyme in rat kidney[7].

Material and methods. The phosphatase source was a fraction of rat kidney homogenate, prepared as follows: the kidney was excised from the anaesthetized rat and immediately homogenized in 10 Vol of 300 m*M* sucrose, 2 m*M* EDTA, 10 m*M* triethanolamine buffer, at pH 7.2; the homogenate was centrifuged at 4000 *g* for 15 min and the supernatant fraction used as enzyme preparation. All operations were carried out at 1–3°C.

p-Nitrophenylphosphate was a B.D.H. product, glycerol-1-phosphate and glycerol-2-phosphate were Sigma products; other chemicals were from B.D.H. or Merck.

Phosphate was determined by the Fiske and Subbarow method[8]. Optical determinations were carried out with an Eppendorf recording photometer, operating with selected spectral lines from a mercury lamp[9].

[1] This research is a contribution from the 'Impresa Enzimologia' Group of the Italian Consiglio Nazionale delle Ricerche.

[2] H. BRANDENBERGER and R. HANSON, Helv. chim. Acta *36*, 900 (1953).
[3] B. H. J. HOFSTEE, Arch. Biochem. Biophys. *51*, 139 (1954).
[4] Y. OHMORI, Enzymologia *4*, 217 (1937).
[5] O. A. BESSEY, O. H. LOWRY, and M. J. BROCK, J. biol. Chem. *164*, 321 (1946).
[6] A. TORRIANI, Biochim. biophys. Acta *38*, 460 (1960).
[7] F. MELANI, G. RAMPONI, A. GUERRITORE, and V. BACCARI, Nature *201*, 710 (1964).
[8] C. H. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 375 (1925).
[9] G. BEISENHERZ, H. J. BOLTZE, TH. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT, and G. PFLEIDERER, Z. Naturforschg. *8b*, 555 (1953).

Principle and optimal test conditions. The splitting of *p*-nitrophenylphosphate by phosphatase activity at alkaline pH results in the liberation of yellow *p*-nitrophenolate, and the increase in extinction at 405 mμ is recorded as a function of time. At this wavelength *p*-nitrophenylphosphate has a negligible extinction[5].

The relation between pH and extinction coefficient of *p*-nitrophenol at 405 mμ is shown in Figure 1. The *p*-nitrophenol is a well known pH indicator, with pK = 7.15 at 25°C[10]; the extinction coefficient is therefore pH-dependent and its value for the calculation of the hydrolyzed *p*-nitrophenylphosphate is taken in accordance with the pH of the enzymatic test.

Optimal conditions for the test were investigated for rat kidney phosphatase. The effects of substrate concentration and pH on enzyme activity are shown in Figure 2. Magnesium concentration is not critical: an optimal and constant activity is obtained for a concentration range between $1.5 \cdot 10^{-4}$ m*M* and 1.2 m*M*.

According to these data the reaction mixture for the phosphatase test contains:

	Volume ml	Final concentration m*M*
200 m*M* glycine buffer, pH 10.4	0.500	100
1.5 m*M* $MgSO_4$	0.100	0.15
150 m*M* Na *p*-nitrophenylphosphate	0.100	15
Enzyme solution	0.010 or more	
Water to	1.000	

The test is carried out in the cell (1 cm light path) of the recording photometer, equipped with a thermoregulated cell holder, at 25°C; wavelength is 405 mμ.

Results. Figure 3 represents the chart of an assay, showing the apparent zero order of the reaction and the proportionality of the rate to the enzyme quantity.

The splitting of 1 μmole of substrate corresponds in the above conditions to an extinction change (ΔE) of 18.0.

According to recommendations of the International Union of Biochemistry[11], enzyme activity is best expressed as μmoles of transformed substrate per min. In order to obtain the μmoles/min of hydrolyzed *p*-nitrophenylphosphate the ΔE/min is multiplied by 0.055.

A comparison of the above procedure with tests using glycerol-1-phosphate or glycerol-2-phosphate as substrate is shown in the Table.

Activity of rat kidney phosphatase on *p*-nitrophenylphosphate, glycerol-1-phosphate and glycerol-2-phosphate. The same enzyme preparation was tested for phosphatase activity at pH 10.2 with the three substrates. Activity with glycerol-phosphates was measured from the phosphate liberation in the following reaction mixtures at 25°C: glycine buffer 100 m*M*; magnesium chloride 1.1 m*M*; substrate 15 m*M*; enzyme solution. Samples for phosphate analysis were collected at zero time and after 10 and 20 min. Activity with *p*-nitrophenylphosphate was measured by the optical technique described

Substrate	Activity (μmoles/min/g wet weight)
p-Nitrophenylphosphate	9.2
Glycerol-1-phosphate	0.8
Glycerol-2-phosphate	2.4

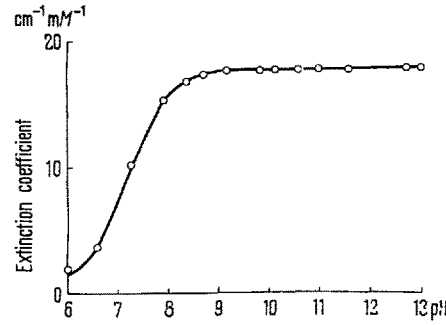

Fig. 1. Extinction coefficient of *p*-nitrophenol in function of pH. Conditions of measurement: *p*-nitrophenol 0.05 m*M* in 100 m*M* glycine, adjusted with NaOH at the pH values indicated. 405 mμ–1 cm cell.

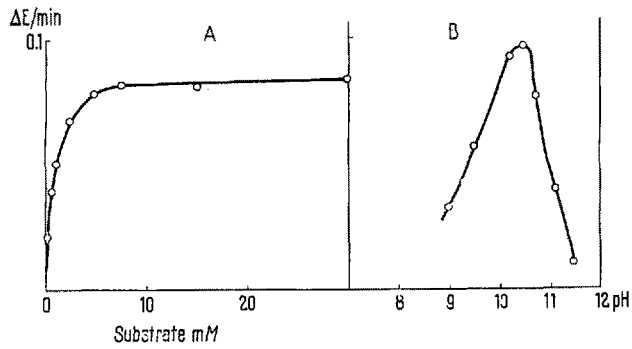

Fig. 2. Effect of substrate concentration (A) and of pH (B) on the activity of rat kidney phosphatase. Test conditions are described in the text: variations of *p*-nitrophenylphosphate molarity or of pH are indicated in the Figure. The fraction of rat kidney homogenate is diluted 1:5 with glycine buffer immediately before addition in the photometer cell: assay starts with enzyme, 25 μl. 405 mμ–1 cm cell–25°C.

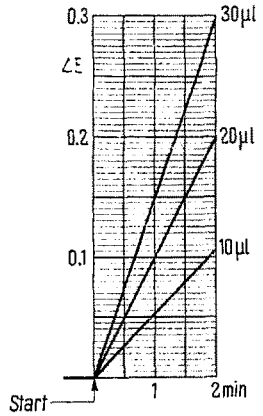

Fig. 3. Optical measurement of rat kidney alkaline phosphatase. Test conditions are described in the text. The fraction of rat kidney homogenate is diluted 1:5 with glycine buffer immediately before addition in the photometer cell: assay starts with enzyme, 10, 20 or 30 μl as indicated in the Figure. 405 mμ–1 cm cell–25°C–chart speed: 20 mm/min.

[10] C. Long, *Biochemists' Handbook* (Van Nostrand Co., Princeton 1961), p. 50.

[11] Report of the Commission on Enzymes of the I.U.B. (Pergamon Press, Oxford 1961), p. 8.

Discussion. In view of the short reaction time and direct control of kinetics, the described continuous optical procedure for phosphatase estimation presents improved accuracy with respect to discontinuous sampling methods, and appears particularly suitable for kinetic studies. The remarkable simplicity of the determination furthermore enables measurements of high precision and reproducibility to be obtained.

The test can be carried out at all pH values in the alkaline range, provided that the appropriate extinction coefficient for the liberated *p*-nitrophenol is used.

Zusammenfassung. Ein kontinuierliches optisches Messverfahren für die Bestimmung der alkalischen Phosphatase mit *p*-Nitrophenyl-phosphat als Substrat wird ausführlich beschrieben. Das Verfahren wird zur Bestimmung der alkalischen Phosphatase in der Rattenniere angewandt.

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Istituto di Chimica Biologica dell'Università di Firenze (Italy), April 17, 1964.

Ophthalmoscopy of Pigeons Using Transillumination

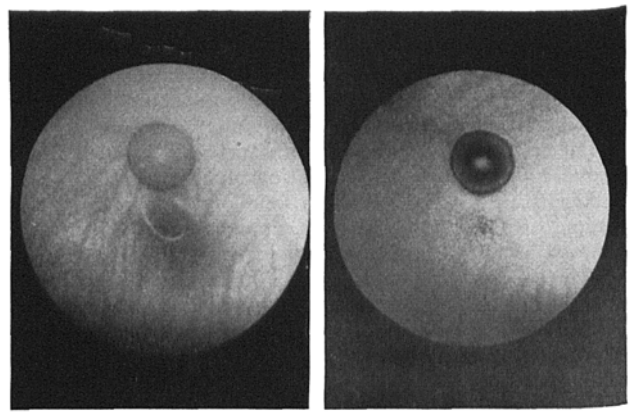
Through the invention of the direct ophthalmoscope by HELMHOLTZ[1], and its subsequent improvement by GULLSTRAND et al.[2], the central areas of the fundus are able to be examined easily. However, only with large pupil sizes are the areas of the peripheral fundus conveniently observed. This is especially true in human subjects whose pupils are dilated conveniently to 6 mm in diameter, or more. However, ophthalmoscopic examination of a bird is not as easily accomplished. The pupil is normally small and, as the iris is skeletal muscle, cannot be enlarged by the usual adrenergic blocking agents. Consequently, poisons such as nicotine or similar ganglionic or neuromuscular blocking agents must be employed with some risk to the life of the animal at concentrations which produce maximal dilation. At best, these produce a pupillary dilation of 5 mm.

Most indirect ophthalmoscopes and cameras are designed for larger pupillary apertures, or even when pupil size is immaterial, as with the Zeiss fundus camera, annoying corneal light reflexes are present. We have developed a different method, which we believe to be useful for both examination and photography of birds and possibly other small animals with eyes on opposite sides of the head. This method is indirect, transverse illumination and is an adaption of those methods used on humans by STEVENSON[3] and others[2] where the illuminating light is placed in the mouth or nose.

Both eyes of the animal are dilated (although this need not be to a maximal degree) by an appropriate cycloplegic agent[4], after which the head is held firmly by some type of holder. Both eyelids are taped open to prevent blinking but the nicitating membrane is left free. A bright light, such as that from an ordinary dissecting lamp, is focused onto the pupil of one eye. (Suitable precautions should be taken to prevent overheating the eye, with subsequent cataract formation.) With this light properly positioned to fall on the pupil, the opposite eye is illuminated by the light passing through the head.

The eye of the observer is brought up to the transilluminated eye and the entire globe can be seen clearly, including that area behind the pecten usually shadowed by the illuminating light from conventional ophthalmoscopes. Also, the light reflex around the fovea is now reversed and the fovea shows up as a dark diffusely granular area.

The examination should be conducted in a darkened room, and a shield to prevent light from the lamp from straying into the observer's eye is also helpful. No practice is necessary; contrary to their first experiences using the ordinary ophthalmoscope, untrained workers can make a good examination of the eye on their first attempt. Conventional fundus cameras will take excellent photographs when transilluminated by a properly triggered flash lamp.



A B

Photographs of a pigeon's eye taken with a Noyori hand fundus camera approximately 40 min after producing a lesion with a Laser flash. In the center of each picture is the fovea with the lesion immediately above it. A was taken using the built-in flash illumination of the camera. B was taken using a flash lamp focused on the pupil of the opposite eye to transilluminate. In A the vessels of the choroidal circulation are quite prominent and there is a light reflex around the fovea. The lesion has very low contrast. In B the choroidal circulation is indistinct, the fovea has no light reflex and shows only as a diffuse dark granular mass, but the lesion has extremely high contrast.

[1] H. v. HELMHOLTZ, *Beschreibung eines Augen-Spiegels zur Untersuchung der Netzhaut im lebenden Auge* (Förstner, Berlin 1851).

[2] For a critical review and history of ophthalmoscopy see W. S. DUKE-ELDER, *Text-Book of Ophthalmology*, Vol. II (Mosby, St. Louis 1937).

[3] N. STEVENSON, Brit. Med. J. *1*, 379 (1893).

[4] For the pigeon, we use several applications of a 10% cocaine solution followed by several applications of a 1% nicotine solution.