Discussion. During the use of this method in routine examination, it was established that the method fulfils the above-mentioned demands. The high sensitivity in the detection of orthophosphate ($6.25 \cdot 10^{-8} M$) allows this method to be used in the estimation of activity of other specific phosphatases. The splitting of the α-glycerolphosphate and glucose-1-phosphate in acetate buffer pH 5.0 and of adenosine-5'-triphosphate in veronal-acetate buffer pH 8.6 were demonstrated. Moreover, a reaction of phenol (15.6 *ng*) with ferric chloride could be used for demonstration of non-specific phosphatase with phenylphosphate as a substrate. The principle could also be used for evidence of a reduction of tetrazolium salts, of the liberation of acids from esters (in a layer containing bromthymol blue), and for evidence of amylase in a layer containing starch. As standards, ammonium sulfide was used for reduction of triphenyltetrazolium chloride and acetic acid for bromthymol blue and the reactions were demonstrable with 125 *ng* and $7.8 \cdot 10^{-8} M$ of standards, respectively. The principle that has been described allows also fast semiquantitative estimation of hemoglobin in minute amounts (8 *ng* and more) in a layer containing benzidine reagent.

Zusammenfassung. Rasche und einfache semiquantitative Methode zur Bestimmung der Aktivität von spezifischen Phosphatasen bei Reihenuntersuchungen: Detektion des Orthophosphats nach Diffusion des Enzyms durch eine Sephadex G-75-Schicht. Auf die Möglichkeiten ihrer Anwendung zur Bestimmung anderer Enzyme wird hingewiesen.

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Purification of Hemocyanin from Hemolymph by Adsorption to Calcium Phosphate

An expedient way of purifying hemocyanin from hemolymph is available if advantage is taken of the adsorption of hemocynin to calcium phosphate. We have used this property to purify hemocyanin from the hemolymph of the whelk, *Murex trunculus*.

Hemolymph was obtained from *M. trunculus* by breaking off the apex of the shell and squeezing the snail inside. 25 ml of this material obtained from 36 snails were made up to 50 ml with $0.01 M$ acetate buffer, pH 5.0, and centrifuged to remove tissue debris. 10 ml of a suspension of hydrated tricalcium phosphate[1] in distilled water (0.1 g/ml) were added to the supernatant. The mixture was stirred and centrifuged to remove the calcium phosphate, which was subsequently eluted with 25 ml $0.1 M$ phosphate buffer, pH 7.0. The pH of the eluate was adjusted to 4.5 with $6 M$ acetic acid, and the solution was half-saturated with ammonium sulphate. The precipitated hemocyanin was separated by centrifuging at 3000 *g* at 4 °C for 30 min, and dissolved in a small quantity of $0.1 M$ phosphate buffer, pH 7.0.

The purification of the hemocyanin during the various stages is given in the Table. A fraction of the material available at each stage was diluted until it had an absorbance of approximately 1.00 at 280 nm in a 1 cm cell. The copper concentration was then determined by the method of PETERSON and BOLLIER[2]. The ratio of copper concentration to absorbance was used as a measure of the purity of the hemocyanin.

It is seen from the Table that 30% purification of the hemocyanin in the starting material was achieved by adsorption to calcium phosphate and elution; a further small degree of purification was obtained by precipitation with ammonium sulphate. This step also served to concentrate the hemocyanin.

The hemocyanin which was obtained migrated towards the anode as one component in agar-gel electrophoresis at pH 7.0. The protein concentration of two preparations was calculated from their nitrogen content, which was determined by a micro-Kjeldahl method[3] after removal of ammonium sulphate by gel filtration through a column of Sephadex G-25 equilibrated with $0.1 M$ phosphate buffer, pH 7.0. It was assumed that the hemocyanin contained 16% nitrogen. These preparations were found to contain 0.26% and 0.27% copper respectively. These values compare with the value of 0.260% reported by GHIRETTI-MAGALDI et al.[4] for hemocyanin of *M. trunculus* prepared by ultracentrifugation of hemolymph.

The adsorption of hemocyanin to hydroxylapatite has been described by other workers[5], but this convenient property has not been used to purify hemocyanins. The

Stage	Copper concentration (μg/ml)	Absorbance (280 nm/ 1 cm cell)	Cu concentration Absorbance	Cu concentration Purification
Diluted hemolymph	1.09	0.94	1.16	1.0
Calcium phosphate eluate	1.45	1.00	1.45	1.3
Ammonium sulphate precipitate	1.66	0.97	1.58	1.4

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present work indicates that hydrated tricalcium phosphate also is an efficient adsorbent of hemocyanin, and its general use in the purification of hemocyanins from their natural sources is suggested.

Résumé. On a purifié l'hémocyanine de l'hémolymphe du Buccin, *Murex trunculus*, principalement par adsorption au phosphate de calcium. On propose l'emploi général de cette méthode de purification des hémocyanines.

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STUDIORUM PROGRESSUS

Timidity and Metabolic Elimination Patterns in Audiogenic-Seizure Susceptible and Resistant Female Rats

Inconsistencies exist concerning the correlations of timidity to emotionality and to audiogenic-seizure susceptibility. It has been reported that emotional rats are non-aggressive and are more timid in the home cage and in the presence of the investigator[1,2]. On the other hand, others found no relationship between timidity, or fearfulness and emotionality[3,4].

Whereas, Martin and Hall[5] and Billingslea[1] concluded that non-emotional rats are more susceptible to audiogenic-seizures, Griffiths[6] conversely obtained results opposed to the hypothesis that non-emotionality and low activity are directly related to seizure-susceptibility. Lindzey[7], likewise, reported a positive relationship between seizure-susceptibility and emotionality.

This investigation sought to clarify the relationship between timidity and audiogenic-seizure susceptibility as well as to study the daily metabolic elimination processes of susceptible versus resistant rats. It is of interest that increases in fecal elimination and urination responses by rats and mice during short time intervals (3–5 min) in open-field enclosures have been reported to be valid measures of emotionality[4,5].

59 female Wistar rats averaging 75 g were tested for susceptibility to audiogenic seizures and convulsions. All rats were subjected to alarm bell stimuli for a maximum of 5 min on 4 occasions during a 2 week preliminary trial and acclimatization period. Intervals of 3–4 days elapsed between the successive auditory-stress trials. Whenever convulsive attacks occurred, the auditory stimulus was stopped immediately to minimize possible stress effects induced by the convulsive process. The mixed-noise levels attained a range of 115–120 decibels depending on the loci of the animals. All test and control rats were housed, tested and stimulated singly in 16 × 18 × 11 in. metal cages. It should be recognized that isolation per se can produce behavioral, metabolic and endocrinal alterations[8].

At the time of the stimulus, the alarm bell was placed centrally on the wire-mesh ceiling of the metal cage. Locomotor and behavioral activities of each rat were observed prior to, during, and for a 5 min post-noise period. The onset and frequency of wild running, tonic and/or clonic convulsions were used as criteria for grouping of the susceptible rats according to the frequency and severity of the audiogenic-seizures and convulsive attacks.

Upon completion of the pre-screening and classification procedures, body weight, food consumption, dry fecal weights and fecal pellet counts were recorded weekly during the following 2 week rest and study period. 24 h urine collections were initiated on the 5th day. As a prerequisite for timidity determinations, all animals were deprived of food and water during the urine collection period. Subsequently, the animals were then transferred to a timidity testing cage similar in size to its home cage.

The degree of timidity of the seizure-susceptible and resistant rats was determined by modifications of Bindra and Thompson[3] and Hunt and Otis[4] procedures. Timidity has been assayed by the amount of time required for a hungry rat to leave its cage and to seize food pellets. The testing cage was located on an illuminated table top with a dish of Purina fox chow 12 inches in front of the cage. Each animal was allowed to acclimatize in the closed cage for 15 min, then the cage door was opened to permit free access to the food supply. The activity and behavior of each animal was then recorded for a 20 min period, in particular, grooming and corner-to-corner exploratory activities, as well as the time needed for the rodent to place both forefect out of the cage, to leave the cage and to reach the food dish. Arbitrary scores of 1200 sec were assigned to animals which did not attain these goals.

While realizing the possibility of errors due to subjective evaluations, to facilitate the determinations of gradations in the response patterns of the animals, all audiogenic-seizure susceptible rats were grouped according to the frequency and severity of the wild-running seizures and convulsive attacks. Group I, for example, contained the most susceptible animals, each rat having circled wildly and suffered a convulsive attack at each of the 4 auditory-stress trials. Group II consisted of animals displaying running seizures at each of the 4 trials but only 2–3 convulsions, and group III, the least susceptible, contained rats exhibiting 1–4 seizures and either none or at most 2 convulsions during the 4 trials. The control group consisted of animals showing complete resistance to the auditory-stress stimuli. None of these rats exhibited signs of wild-running seizures or convulsions.

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