Effects of Oxygen on Determination of Thiocholine by 5,5′-Dithiobis(2-Nitrobenzoic Acid).* (31047)

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DTNB (5,5′-dithiobis-2-nitrobenzoic acid) is widely used with thioesters of choline for cholinesterase assays according to the method of Ellman *et al*(1,2). Whether the hydrolysis is enzymatic or spontaneous, one of the products is thiocholine. Thiocholine reacts with an excess of DTNB in a sulfide-disulfide exchange reaction to yield 2 products: a mixed disulfide and TNB (5-thio-2-nitrobenzoic acid). For example, when cysteine is added to a 30-fold molar excess of DTNB, a 1:1 molar exchange reaction yields a stoichiometric equivalent of TNB, which is colored. By using this reaction (equation 1 below), we have confirmed that the molar extinction coefficient of TNB is 1.36×10^4 at 412 mμ. If cysteine is present in a 30-fold excess, the reaction goes one step further to produce 2 moles of TNB, as evidenced by a color with the same spectral characteristics but twice the intensity (equation 2).

1. $$\underset{\text{(cysteine)}}{\text{R-SH}} + \underset{\text{excess}}{\underset{\text{(DTNB)}}{\text{Ø-S-S-Ø}}} \longrightarrow \underset{\text{(mixed disulfide)}}{\text{R-S-S-Ø}} + \underset{\text{(TNB)}}{\text{Ø-SH}}$$

2. $$\underset{\text{(cysteine)}}{\underset{\text{excess}}{\text{R-SH}}} + \underset{\text{(DTNB)}}{\text{Ø-S-S-Ø}} \longrightarrow \underset{\text{(cystine)}}{\text{R-S-S-R}} + \underset{\text{(TNB)}}{\text{2Ø-SH}}$$

Although DTNB is widely employed for cholinesterase assays, difficulties were encountered in using it to follow the slow spontaneous hydrolysis of thiocholine esters. With procedural modifications described in this report, however, DTNB is eminently satisfactory for this purpose.

Methods. Phosphate buffer, 0.1 M, pH 8.0 (33.52 g $Na_2HPO_4 \cdot 12\ H_2O$ and 0.86 g KH_2PO_4 per liter); DTNB (Aldrich Chemical Co., Inc.), 0.01 M stock solution prepared by dissolving 39.6 mg in 10 ml phosphate buffer; benzoylthiocholine iodide (Dajac Laboratories); acetylthiocholine iodide (Nutritional Biochemicals Corp.); L-cysteine, free base; alkaline pyrogallol, 150 g anhydrous KOH dissolved in 100 ml water and nitrogenated for 10 minutes in a gas scrubber followed by the addition of 10 g pyrogallic acid.

Thunberg photometric tubes, either square tubes with a 1 cm light path for UV experiments or cylindrical tubes with an internal diameter of 1.65 cm for colorimetric determinations; spectrophotometer, either the Zeiss PM Q II for the UV range or the Beckman model B for the visible range.

Because the stability of sulfhydryl compounds is known to be influenced by low concentrations of heavy metals, all glassware was washed with nitric acid and rinsed with doubly distilled water (Scorah still). More elaborate precautions to insure water purity were regarded as inappropriate because no information is available about possible metal contamination of several essential reagents listed above. DTNB and benzoylthiocholine stock solutions were always freshly prepared just prior to use. Phosphate buffer for use in anaerobic experiments was deoxygenated by bubbling oxygen-free nitrogen (treated with alkaline pyrogallol) for at least 30 minutes prior to the start of each experiment. Benzoylthiocholine stock solution, *e.g.*, 0.005 M, was placed in the cap bulb of the Thunberg tube. To 9.0 ml of phosphate buffer in the main chamber was added DTNB, *e.g.*, 0.3 ml of 0.01 M to give a final concentration of 3.2×10^{-4} M. Pyrex glass boiling chips were added before sealing. While incubating in a constant temperature bath, each tube was evacuated and flushed with nitrogen or air. Evacuation and flushing were repeated over a 15-minute period. Tubes for anaerobic experiments were then sealed; tubes for aerobic experiments were left exposed to the air. Prior to reading, the tube contents were mixed by inversion. Anaerobic tubes were read against appropriate anaerobic blanks and aerobic tubes against aerobic blanks.

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TABLE I. Apparent Amount of Thiocholine Released (Micromoles per Liter) at 25°C During 30 Minutes of Spontaneous Hydrolysis of 500 μM Benzoylthiocholine in Five 0.1 M Buffer Systems at pH 8.0.*

Phosphate	Imidazole	Pyrophosphate	Barbital	Glycylglycine
65.1 ± 3.6	78.6 ± 59.3	80.2 ± .9	114 ± 6.6	152 ± 1.0

* Experiments performed in air-saturated solutions in the presence of DTNB. Values are means and standard deviations for at least 5 replicate determinations.

For UV experiments DTNB was omitted; otherwise the procedure was the same as described above. Fluid volumes were one-third those in the colorimetric determination. At pH 8.0 absorption maxima of benzoic acid and benzoylthiocholine are 224 and 231 mμ respectively. The UV data in Fig. 2, however, are based on measurements at 266 mμ. At 266 mμ benzoic acid does not absorb, whereas the molar extinction coefficient of benzoylthiocholine is about 1.3×10^4.

Results. According to colorimetric measurements in the presence of DTNB, benzoylthiocholine proved to be unstable at 25°C in five 0.1 M buffer systems: glycylglycine, pyrophosphate, barbital, phosphate and imidazole (Table I). The data suggest specific anion influences on the rates of spontaneous hydrolysis. Hydrolysis was slowest but still appreciable in phosphate buffer. The results obtained with imidazole were so erratic as to defy interpretation. Because molecular oxygen was not excluded, the true rates may have been appreciably higher in some cases, as noted below.

In deoxygenated solutions with DTNB present throughout, the first order rate constants for spontaneous hydrolysis of benzoylthiocholine in 0.1 M phosphate buffer at 25° were 0.23 hour^{-1} at pH 8.0 and 0.03 hour^{-1} at pH 6.8 (Fig. 1). At pH 8 the corresponding value for acetylcholine was 0.005 hour^{-1}, as estimated from a hydrolysis period of 20 hours. The rate of spontaneous hydrolysis of benzoylthiocholine could be decreased by further reductions in pH, *e.g.*, hydrolysis was almost negligible in a 0.0625 M stock solution at pH 4.8 when stored at 5°.

Data on the left in Fig. 2 suggest that the rate of hydrolysis is dependent on the oxygen tension. Here benzoylthiocholine in alkaline phosphate buffer was incubated at 25° in the presence and absence of air. Thiocholine was estimated by removing aliquots and adding DTNB to them just before each reading (412 mμ). Essentially no thiocholine was detected in the aerobic solution, whereas it accumulated progressively in the anaerobic system. However, when the solutions were analyzed for benzoylthiocholine by UV absorbance at 266 mμ (Fig. 2 on the right), it was apparent that hydrolysis occurred in both solutions at the same rate. Apparently in the presence of oxygen thiocholine is rapidly oxidized, presumably to the disulfide, and so is unavailable for reaction with DTNB. The absence of air is seen to stabilize thiocholine as the monothiol. These results led to an examination of the stabilities of thiocholine, TNB, and DTNB in solution.

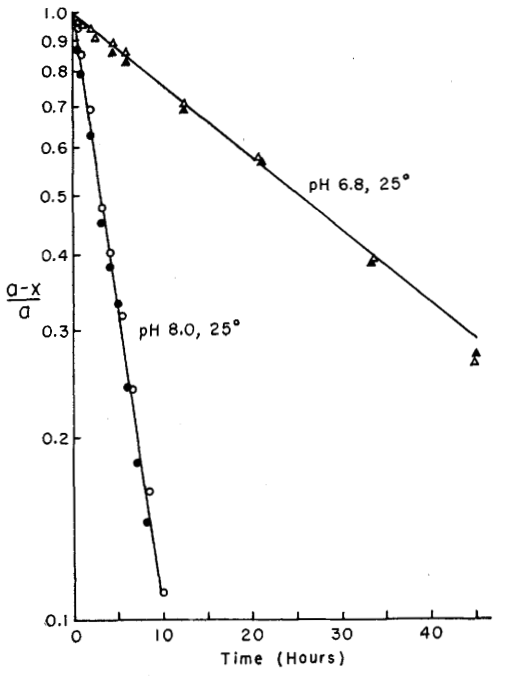


FIG. 1. Spontaneous hydrolysis of 4×10^{-5} M benzoylthiocholine at 25° in 0.1 M deoxygenated phosphate buffer at pH 6.8 or 8.0. Symbols of the same shape are duplicate reaction mixtures.

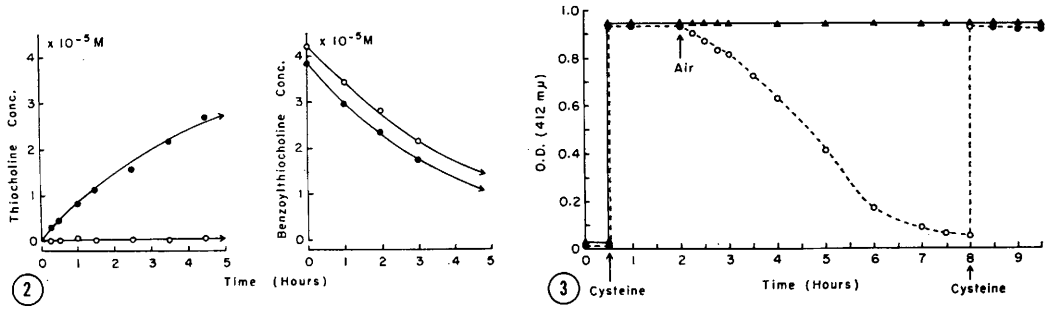


FIG. 2. Spontaneous hydrolysis at 25° of benzoylthiocholine (initial conc. approx. 4 × 10^{-5} M) in 0.1 M phosphate buffer at pH 8.0. Open symbols represent aerobic solutions and solid symbols anaerobic solutions. On the left thiocholine was estimated by adding DTNB to aliquots removed from the reaction mixtures just before each reading. On the right benzoylthiocholine concentrations were estimated by the absorbance at 266 mμ (molar extinction coefficient 1.3 × 10^4).

FIG. 3. On the left are recorded the absorbance of 2 solutions of buffered DTNB (2.1 × 10^{-5} M) sealed anaerobically in Thunberg tubes. After 30 minutes TNB was generated by tipping in a 2-fold molar excess of cysteine stored in the bulb cap. One tube remained anaerobic for 10 hours (solid triangles). The other was exposed to air after 2 hours (open circles), and excess cysteine was re-added at 8 hours.

Fig. 3 shows that an anaerobic solution of TNB, generated by adding excess cysteine to DTNB in phosphate buffer, was stable for at least 10 hours. When the solution was aerated, however, TNB was auto-oxidized to DTNB, the visible result being a loss of color. With less cysteine present, fading occurred much faster; presumably unreacted cysteine tends to stabilize TNB by competing with it for dissolved oxygen. When cysteine was re-added to the aerated solution (Fig. 3), the original TNB color was immediately and fully regenerated, demonstrating the reversibility of this oxidation.

At high temperatures the DTNB dimer-monomer system is not fully reversible. As shown in Fig. 4 dilute solutions of DTNB in phosphate buffer were incubated at various temperatures under anaerobic and aerobic conditions. At 25° and 37° there was no appreciable reduction of DTNB to TNB, but at 75° and 90° color formation was distinct. Fig. 4 summarizes the TNB equivalent of this color (412 mμ), although it is possible that more than one reaction product contributed to the absorbance. After 2 hours excess cysteine was added to each solution. In aerobic and anaerobic solutions at 25° and 37°, TNB was promptly produced in an amount equivalent to the original DTNB. At 75° and 90° cysteine failed to generate the full TNB color, even when the solutions were allowed to cool. Thus heat displaces the DTNB:2TNB equilibrium and also irreversibly damages one or both components of the system. Fig. 4 suggests that this thermal damage is greater in air than in nitrogen.

With higher concentrations of DTNB (32

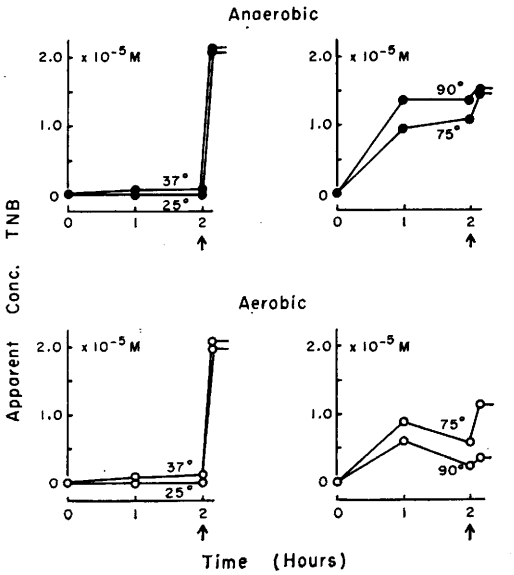
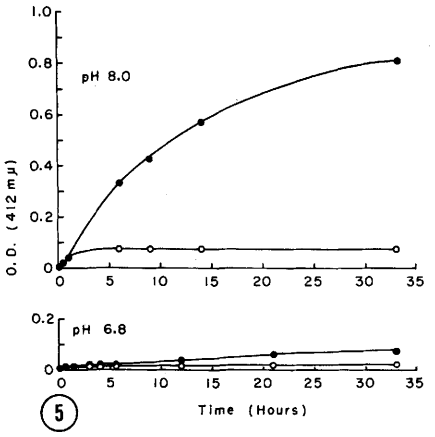


FIG. 4. Effects of heat and air on equilibrium and reversibility of the DTNB:2TNB system. Identical solutions of 1.07 × 10^{-5} M DTNB in pH 8.0 phosphate buffer were incubated at 4 temperatures with and without air. Excess cysteine (1.07 x 10^{-4} M) was added after 2 hours (at arrows). TNB concentrations were inferred from the absorbance at 412 mμ.



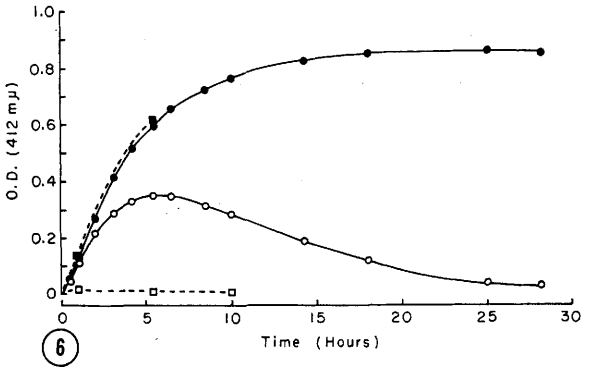


FIG. 5. Effects of pH and air on spontaneous reduction of DTNB and equilibrium of the DTNB: 2TNB system at 25°. Open symbols show readings in presence of air; solid symbols are anerobic readings. At zero time all solutions consisted of 3.2×10^{-4} M DTNB in phosphate buffer.

FIG. 6. Long term effects of air on DTNB determination of thiocholine released by spontaneous hydrolysis of benzoylthiocholine (4×10^{-5} M) in phosphate buffer at pH 8. Open symbols denote air-saturated solutions, solid symbols indicate anaerobic solutions. Circles represent solutions in which DTNB was present throughout the entire experiment. Squares represent trials in which DTNB was added to aliquots of the benzoylthiocholine solution just prior to each reading.

$\times 10^{-5}$ M) spontaneous reduction to TNB can be demonstrated even at 25°. The mechanism of this reaction is unknown, but the product has the spectral characteristics of genuine TNB. Fig. 5 shows that spontaneous splitting of the disulfide bond occurred much more rapidly at pH 8.0 than at pH 6.8. It also appeared to be faster when oxygen was excluded, presumably because oxygen tends to oxidize TNB back to the dimer. Even in an anaerobic solution at pH 8 (Fig. 5) the highest concentration of TNB attained represented only a 5% conversion of DTNB. At pH's below 7 reduction was negligible over periods of several hours. Because DTNB repeatedly recrystallized from methanol showed similar rates of reduction, an impurity was probably not involved in this phenomenon.

Various procedures were tested to discover optimal conditions for using DTNB in thiocholine assays. In the experiments of Table II the amount of thiocholine released was significantly underestimated by the conventional DTNB method. The first column of Table II shows the optical density obtained when DTNB was present throughout 30 minutes of spontaneous hydrolysis of benzoylthiocholine in a deoxygenated solution. The mean is significantly higher ($p<0.01$) than that observed with air-saturated solutions (column 2). However, when the solution was deoxygenated and DTNB added at the end of 30 minutes (column 3), the results were not significantly different from column 1. When the solution was air-saturated and DTNB was omitted (column 4), its addition at the end of 30 minutes revealed that less than 10% of the thiocholine released was available for reaction. This effect of oxygen is greatly magnified when the time period of the hydrolysis is increased (Fig. 6).

Discussion. The results may now be examined in relation to the utility of DTNB for measuring the hydrolysis of thiocholine esters. In using this reagent one must obviously minimize thiocholine losses through auto-oxidation to dithiocholine (thiocholine disulfide) and to any other products that do not react with DTNB. As seen in Fig. 6, oxidative losses of thiocholine in air-saturated solutions are almost complete if DTNB is absent. In the usual analytical procedure DTNB is present throughout the hydrolysis (2). If air is also present, DTNB must compete with molecular oxygen for thiocholine as it is released. After 30 minutes of hydrolysis significantly more thiocholine is demonstrable by the DTNB method in deoxygenated than in air-saturated solutions (Table II), and the difference increases progressively

TABLE II. Optical Density Produced During 30 Minutes of Spontaneous Hydrolysis of 3.3×10^{-4} M Benzoylthiocholine.*

DTNB present throughout hydrolysis		DTNB added at end of hydrolysis	
Anaerobic	Aerobic	Anaerobic	Aerobic
.82 ±.016	.74 ±.035	.84 ±.032	.08 ±.045

* Means and standard deviations for 6 replicate determinations.

thereafter (*cf.*, open and solid circles in Fig. 6). This difference represents not only losses of thiocholine by auto-oxidation but also color fading due to the slow oxidation of TNB in the air-saturated solutions of Fig. 3 and 6.

In experiments of only a few hours duration, the hydrolytic release of thiocholine can be monitored without significant error by using DTNB at the recommended concentration *if* oxygen is carefully excluded. Under these circumstances, however, appreciable amounts of TNB may be generated after several hours by the spontaneous reduction of DTNB (Fig. 5). Whatever the mechanism of this reaction, it can be corrected for by using an appropriate anaerobic blank (as in Fig. 6) or by adding DTNB only to aliquots of the anaerobic reaction mixture just prior to each reading (also as in Fig. 6). At pH's below 7 reduction is so slow that no correction is required (Fig. 5). Ellman *et al*(2) were aware that DTNB solutions are unstable at pH 8 and suggested that stock solutions be stored at pH 7.

The ready oxidizability of many monothiols in solution has long been recognized. Hansen (3) suggests that auto-oxidation is most rapid for sulfhydryl compounds that are present as thiolate anions; alkaline solutions are therefore more unstable. That thiocholine is rapidly oxidized in aqueous solutions has been mentioned by various investigators(3,4,5), but the necessity for excluding air has not been emphasized or adequately demonstrated (6). From the average optical densities recorded in the two right-hand columns of Table II, one can estimate that thiocholine had a half-life of approximately 5 minutes in these air-saturated solutions.‡ Similar values were obtained in other trials with other initial concentrations. This demonstration has obvious relevance to thiocholine analyses whether by the DTNB method or by other colorimetric (7), histochemical(8), electrochemical(4,9), or pharmacological methods(5). Even though the amount of error, if any, introduced in these various procedures by the failure to exclude oxygen cannot be inferred from the present data, it is likely that oxidation products were responsible for some phenomena that have been ascribed to thiocholine(10).

Because of its rapid alkaline hydrolysis in buffered solutions (Fig. 1), benzoylthiocholine has limited usefulness as an enzyme substrate. In this respect it appears to differ considerably from benzoylcholine, whose half-life in sodium phosphate solutions at pH 8 is estimated by extrapolation to be several weeks (11). This disparity is in conflict with the generalization that thioesters tend to be more stable than their oxy-analogues(12,13). Perhaps the dilemma can be explained by the greater susceptibility of thioester hydrolysis to catalysis by nucleophilic buffer ions. In this laboratory benzoylthiocholine also proved to be less stable than acetylthiocholine in buffered solutions. By the anaerobic DTNB method described above, the half-life of benzoylthiocholine at pH 8 and 25° amounted to 3 hours, whereas that of acetylthiocholine extrapolated to more than 100 hours. Although conditions were not strictly comparable, the former value is not inconsistent with observations by Heilbronn(14); the latter accords with a statement by Ellman *et al*(2). There is no general agreement, however, about the stability of acetylthiocholine. Heilbronn(12, 14) insists that it hydrolyzes much faster than all other thiocholine esters tested, including benzoylthiocholine. At least in part her conclusions are based on titrimetric data, al-

‡ This estimate is possible because of the demonstration that the rate of benzolythiocholine hydrolysis in these relatively concentrated solutions is independent of the oxygen tension and essentially constant for the first 30 minutes. Implicit in the calculation are 2 assumptions: (1) that the DTNB reaction is fast relative to the auto-oxidation of thiocholine, and (2) that the auto-oxidation is first-order with respect to the thiocholine concentration. These assumptions are almost certainly valid, but no precise estimate of the rate constant is possible because of scatter in the data.

though this method is later acknowledged(10) to be inappropriate because of the buffer capacity of thiocholine and its oxidation to secondary products. Further studies are required to clarify the relative stabilities of various thiocholine esters, particularly in the presence of various buffer anions.

Summary. The DTNB (5,5′-dithiobis-2-nitrobenzoic acid) method has been recommended for measuring the hydrolysis of thiocholine esters. During studies on the alkaline hydrolysis of benzoylthiocholine, however, analytical errors have been encountered and traced to the presence of molecular oxygen. Although oxygen does not influence the rate of benzoylthiocholine hydrolysis, it does compete with DTNB for the hydrolysis product thiocholine. Furthermore it produces fading of the chromogen on which the assay is based by oxidizing the monothiol TNB back to the DTNB. Thus the amount of thiocholine released is significantly underestimated if hydrolysis periods of 30 minutes or more are employed. In the absence of oxygen DTNB can be added at any time during the hydrolysis, but in the procedure recommended here it is present throughout. This modified DTNB method was used to determine rate constants for auto-oxidation of thiocholine and for spontaneous alkaline hydrolysis of acetyl- and benzoylthiocholines. Unlike acetylthiocholine, benzoylthiocholine proved to be so unstable in various buffered solutions at pH 8 that it appears to have only limited usefulness as an enzyme substrate.

1. Ellman, G. L., Arch. Biochem. Biophys., 1958, v74, 443.
2. Ellman, G. L., Courtney, K. D., Valentino, A., Featherstone, R. M., Biochem. Pharmacol., 1961, v7, 88.
3. Hansen, B., Svensk. Kem. Tidskr., 1963, v27, 511.
4. Fiserová-Bergerová, V., Collection Czech. Chem. Commun., 1962, v27, 693.
5. Scott, K. A., Mautner, H. G., Biochem. Pharmacol., 1964, v13, 907.
6. Smissaert, H. R., Science, 1964, v143, 129.
7. Gal, E. M., Roth, E., Clin. Chim. Acta, 1957, v2, 316.
8. Koelle, G. B., Cholinesterases and Anticholinesterase Agents, Handbuch der Experimentellen Pharmakologie XV, Springer-Verlag, Berlin, 1963, pp. 191-197.
9. Guilbault, G. G., Kramer, D. N., Cannon, P. L., Jr., Anal. Biochem., 1963, v5, 208.
10. Heilbronn, E., Acta Chem. Scand., 1959, v13, 1547.
11. Kalow, W., Genest, K., Staron, N., Can. J. Biochem. Physiol., 1956, v34, 637.
12. Heilbronn, E., Acta Chem. Scand., 1958, v12, 1481.
13. Schaefgen, J. R., J. Am. Chem. Soc., 1948, v70, 1308.
14. Heilbronn, E., Acta Chem. Scand., 1958, v12, 1492.

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Studies of Hamycin on Inflammation and Related Mechanism. (31048)

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Hamycin,* a heptaene antibiotic(1), has been reported to be a very active antifungal agent against superficial(2) and deep mycoses(3). Following the preliminary report of marked antiinflammatory activity of hamycin on rats(4) it was decided to study its action in detail on inflammation and also on thymus and adrenals which are intimately connected with regulation of such non-specific body defenses.

Experimental. Animals. Male albino rats (Haffkine strain), weighing 100-120 g were starved overnight. The animals were grouped (5-10 per group) at random in all the experiments. In each experiment, a control group containing the same number of animals as the treated group were taken. A minimum total of 15 treated as well as control rats

* Hamycin powder, manufactured by Hindustan Antibiotics Ltd., Pimpri, Poona.