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.

Received November 23, 1965. P.S.E.B.M., 1966, v122.

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

C. V. DAVE, F. F. COUTO AND S. H. AMBIKE (Introduced by V. M. Doctor)
Research Laboratories, Hindustan Antibiotics Limited, Pimpri, Poona

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.

were used for testing each criterion. The results submitted, however, refer to only one typical experiment.

Adrenalectomy was performed on animals under light ether anaesthesia by the usual procedure(5). The animals were kept on normal diet and were given 1% sodium chloride for drinking instead of tap water, until further use.

Drugs tested. In all experiments, varying doses of hamycin (200 mesh powder) were administered with dose volume of 5 ml/kg orally or intraperitoneally as a suspension in normal saline containing 0.1% polysorbate 80.† To compare antiinflammatory activity by the oral route, hydrocortisone‡ and phenylbutazone§ were administered in nearly equipotent doses in the same manner as the corresponding hamycin treated group.

Egg albumin and formalin inflammation. Freshly prepared solutions of egg albumin (2%) and formalin (3%) in normal saline were used to produce inflammation in rats. Animals in each group were injected with the respective inflammatory agent (0.1 ml) in the subplantar region of left paw. Paw volume was measured by the method of Harris and Spencer(6) half an hour before the subplantar injection and subsequently at fixed time intervals. Inflammation was measured as increase in paw volume before and after injection of the irritant.

In groups of rats receiving egg albumin, a rapid onset of the paw inflammation was observed to occur, followed by relatively rapid decrease, whereas in the case of formalin receiving groups, the onset and especially the disappearance were considerably slower. Maximum inflammation was noted between 1-2 hours after egg albumin injection. Statistical comparison of anti-inflammatory responses due to different drugs was made during this period of maximum inflammation.

Hamycin was administered orally (2-200 mg/kg) in 2 divided doses, namely, 20 hours and 4 hours prior to the injection of inflammatory agents, whereas, intraperitoneally, it was given 24 hours earlier as a single dose (3 mg/kg). Hydrocortisone (40 mg/kg) and phenylbutazone (200 mg/kg) were also orally given in the same way as oral hamycin. Hamycin (200 mg/kg) was also orally tested on adrenalectomized rats for its antiinflammatory activity.

Cotton pellet granuloma. The method was essentially based on that reported by Meier *et al*(7). Cotton pellets (7-8 mg) accurately weighed and not differing by more than ± 0.2 mg from one another in any one experimental setup, were used for subcutaneous implantation. Two pellets, one on each lateral side, were implanted through a single longitudinal middorsal incision. Rats were killed with chloroform at the end of the 7th day, pellets with granuloma were removed, dried at 60°C for 24 hours and weighed. Granuloma weight was recorded in milligrams as the increase in the weight of pellets after drying.

Animals were given, orally, 50 mg/kg of hamycin from the first day of implantation till the day prior to removal of the pellets. Hydrocortisone (20 mg/kg) was also given for the same period to other groups of animals.

Effects on body weight, adrenal and thymus. Groups of rats were given 50 mg/kg of hamycin orally each day for one to 7 days or 3 mg/kg of hamycin intraperitoneally on alternate days for 3 days only. Percentage change in the initial body weight of the individual animal at the end of the testing period was considered as growth of the animal. The mean difference between the growth of treated and control groups was taken as the criterion for judging the percentage change in growth due to the drug. The animals were killed with chloroform at the end of the testing period. Thymus and adrenals were separated. The wet glands were weighed immediately and their weights in mg per 100 g of final body weight were calculated. Considering these weights of thymus and adrenals 100% for the control group, the percentage changes in the organ weights for the corresponding treated groups were determined. Adrenals

† Polysorbate 80, Atlas Chemical Co., Inc., Delaware.

‡ Hydrocortisone acetate, 'Efcorlin Suspension' 25 mg/ml M/s Glaxo Laboratories (India) Ltd., Bombay.

§ 'Butazolidin' tablets (200 mg) Suhrid Geigy (India) Ltd., Baroda.

TABLE I. Effect of Hamycin on Maximal Rat Paw Inflammation.

Route	Hamycin Total dose (mg/kg)	No. of rats	Inflammatory agent	Mean increase in paw vol ± S.E. (ml × 100)	% Inhibition of inflammation
Oral	0	5	Egg albumin	63 ± 4	.0
"	2.0	5	"	59 ± 2	6.3
"	20.0	4	"	49 ± 3*	22.2*
"	200.0	5	"	45 ± 5*	28.6*
Oral	0	5	Formalin	46 ± 2	.0
"	2.0	5	"	43 ± 3	6.5
"	20.0	4	"	41 ± 2	10.9
"	200.0	5	"	33 ± 1*	28.3*
I.P.	0	5	Egg albumin	41 ± 5	.0
	3.0	5	"	26 ± 3*	36.6*
"	0	5	Formalin	49 ± 5	.0
	3.0	5	"	23 ± 4*	53.0*

* Values significantly lower ($P < .05$) than the corresponding controls.

were also analyzed for ascorbic acid content (AAA) as described by U.S.P.(8). Percentage changes in both the total values and mg per 100 g of adrenal values of AAA due to hamycin treatment were obtained on the basis of the corresponding control values as 100%.

Results. Egg albumin and formalin inflammation. In rats treated with 2-200 mg/kg of oral hamycin, both types of inflammatory response remained below their respective control levels throughout the testing period. In the case of formalin inflammation the testing period was prolonged as much as 48 hours (Fig. 1). Inhibition of maximal inflammation was, however, significant in groups receiving 200 mg/kg of hamycin (Table I). A similar effect against inflammation due to egg albumin was also observed in the group receiving 20 mg/kg of hamycin (Table I). As can be observed from Table I, the slope of the dose-response curve between doses of oral hamycin and percentage inhibition of inflammation also appears quite low. Intraperitoneally with 3 mg/kg of hamycin both types of inflammatory response were considerably inhibited (Table I).

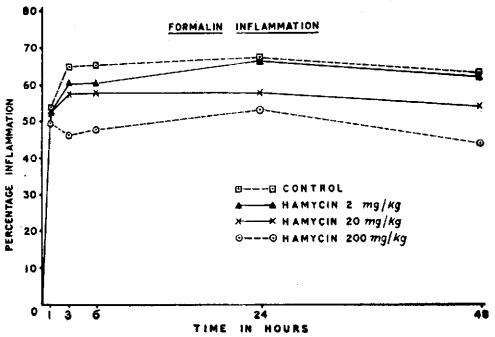

FIG. 1.

Hamycin (200 mg/kg), hydrocortisone (40 mg/kg) and phenylbutazone (200 mg/kg), all given orally, significantly inhibited formalin inflammation (Table II), whereas only the former two inhibited egg albumin inflammation (Table II).

In adrenalectomized rats receiving hamycin (200 mg/kg), the inflammation produced by egg albumin remained unaffected.

Cotton pellet granuloma. Oral hamycin (50 mg/kg/day) in rats significantly prevented granuloma formation around cotton pellets (Table III). Hydrocortisone (20 mg/kg/day) produced somewhat more granuloma inhibition under similar conditions (Table III). Almost similar results were obtained when the experiment was terminated at the end of 4 days instead of the usual 7.

Action on body weight, thymus and adrenal. Pronounced thymolytic activity and adrenal hypertrophy were observed in rats given oral hamycin (50 mg/kg/day) for 3 days or more (Table IV). Comparatively, in groups of animals receiving hamycin there was a gradual decrease in growth depending on the number of days of treatment (Table IV). In rats treated with intraperitoneal hamycin (3 mg/kg) on alternate days, similar but more re-

TABLE II. Effects of Orally Administered Hamycin (HM), Hydrocortisone (HCT) and Phenylbutazone (PHB) on Maximal Rat Paw Inflammation.

Group	Total dose (mg/kg)	No. of rats	Inflammatory agent	Mean increase in paw vol ± S.E. (ml × 100)	% Inhibition of inflammation
Control	0	5	Egg albumin	58 ± 4	.0
HM	200.0	5	"	34 ± 3*	41.4*
HCT	40.0	5	"	41 ± 3*	29.3*
PHB	200.0	4	"	49 ± 3	15.5 (P 0.1)
Control	0	5	Formalin	41 ± 2	.0
HM	200.0	5	"	31 ± 2*	24.4*
HCT	40.0	5	"	27 ± 3*	34.1*
PHB	200.0	5	"	28 ± 4*	31.7*

* Values significantly lower ($P < .05$) than the corresponding controls.

TABLE III. Effect of Orally Administered Hamycin (50 mg/kg, Daily, for 7 Days) and Hydrocortisone Acetate (20 mg/kg, Daily, for 7 Days) on Formation of Cotton Pellets Granuloma in Rats.

Group	No. of animals	Mean initial wt (g)	Mean wt of pellet (mg)	Mean dry granuloma wt ± S.E. (mg)	% Inhibition granuloma formation
Control	10	85.4	7.5	34.4 ± .9	—
Hamycin	8	88.6	7.5	26.9 ±1.2*	21.8*
Hydrocortisone	10	89.3	7.4	23.4 ± .9*	32.0*

Two pellets were implanted in each rat.
* Values significantly lower ($P < .05$) than the corresponding controls.

markable changes were observed as compared to the orally treated group (Table V).

A gradual increase in total AAA occurred in rats after oral and intraperitoneal hamycin administration depending on number of days of treatment (Tables IV and V). The AAA calculated on the basis of mg % weight of adrenals was also found to have increased (Table IV) in orally treated animals, whereas it decreased in intraperitoneally treated animals (Table V).

Discussion. The long sustained antiinflammatory activity observed with oral hamycin against egg albumin- and formalin-induced inflammation was almost comparable to that observed with ⅕ the dose of hydrocortisone and the same dose of phenylbutazone. The granuloma inhibition by hamycin was found to be somewhat less than ⅖ dose of hydrocortisone. Considering the difference in activity between oral and intraperitoneal doses, the relatively low order of activity and the dose response slope with oral hamycin could be due to limited absorption of the drug through the alimentary canal(9,10).

On thymus and body weight, hamycin produced similar effects as do glucocorticoids (11). The antiinflammatory effect, together with these effects, suggest that the cortisone-like activity of hamycin may be due to release of cortisone through the adrenal cortex. This is also supported by the evidence that no antiinflammatory response to the drug was observed in adrenalectomized rats.

Glucocorticoids are known to produce adrenal atrophy by suppression of adrenocorticotrophic hormone (ACTH) of the anterior pituitary(11). Hamycin on the other hand, produced the opposite effect, namely, adrenal hypertrophy along with an increase in AAA. Chronic and increased release of ACTH is reported to cause adrenal hypertrophy as well as increase AAA(12). The action of hamycin, therefore, appears to be analogous to the chronic and increased release of ACTH. This may be due to direct chronic stimulation of the hypothalamic-hypophyseal regions. Indirect proof of the ability of hamycin to cross the blood brain barrier has been given by some workers(13,14) while studying the eradication of *Cryptococcus neoformans* from the brains of mice, and by others(9) studying the toxicity of hamycin. Prolonged antifungal blood levels obtained even with a single oral

TABLE IV. Effect of Oral Hamycin (50 mg/kg, daily) on Thymus and Adrenal of Rat.

Exp No.	No. of days of feeding	Mean percentage changes in treated animals ± pooled S.E.				
				Adrenal		
					Ascorbic acid	
		Growth	Wt of thymus	Wt	Total	mg % of adrenal
1	1	— .4 ± 3.8	— 7.7 ± 8.3	+ .6 ± 4.9	+ 2.7 ± 9.9	+ 9.0 ± 10.1
2	3	—19.1 ± 2.1*	—20.9 ± 5.6*	+20.9 ± 8.8*	+24.4 ± 13.1	+12.8 ± 10.5
3	7	—33.1 ± 1.9*	—41.9 ± 12.3*	+40.6 ± 7.6*	+44.3 ± 6.8*	+42.7 ± 7.6*

Each result is based on 6 control and 6 treated animals.
+ = increase, — = decrease.
* Significantly different ($P < .05$) changes.

TABLE V. Effect of Intraperitoneal Hamycin (3 mg/kg, alternate days) on Thymus and Adrenal of Rat.

Exp No.	No. of days of treated	Mean percentage changes in treated animals ± pooled S.E.				
				Adrenal		
					Ascorbic acid	
		Growth	Wt of thymus	Wt	Total	mg % of adrenal
1	1	— 3.5 ± 4.0	—28.0 ± 20.6	+ 31.2 ± 13.8*	+ 1.0 ± 7.5	—14.7 ± 8.8
2	3	—27.0 ± 2.3*	—65.4 ± 16.5*	+115.0 ± 15.7*	+58.9 ± 4.5*	—10.7 ± 7.9

Each result is based on 6 control and 6 treated animals.
+ = increase, — = decrease.
* Significantly different ($P < .05$) changes.

or systemic dose of hamycin, as reported earlier(10), indicate the availability of the drug for chronic stimulation of appropriate brain centres controlling the release of ACTH.

The only inconsistency in the above hypothesis as to the mechanism of action of hamycin is that in the intraperitoneally treated rats a decrease in mg % content of AAA was observed in spite of a considerable total increase of the AAA. This could be explained as the result of a much greater enlargement of adrenals as compared to the increase in total AAA, resulting in a net decrease in the mg % of AAA. The increase in adrenal weight as compared to the increase in its AAA content could be due to the effect of the maximum tolerated intraperitoneal dose of hamycin employed. This in turn may cause a continuous and excessive liberation of ACTH, resulting in adrenal hypertrophy at a rate exceeding that of regeneration of AAA. No such differential enlargement of adrenals was, however, observed with oral hamycin, possibly because of its slow and limited absorption through the gastro-intestinal tract (9,10). This would, therefore, not allow the blood level to increase as much and as quickly as could possibly be achieved by intraperitoneal hamycin. Delay in achieving the height and rate of blood levels by the oral route as against the intraperitoneal route would also allow time for the adrenal cortical cells to maintain the AAA regeneration in keeping pace with the glandular enlargement.

Summary. Hamycin, a heptaene antifungal antibiotic, showed antiinflammatory activity in rats by oral and intraperitoneal route when tested against paw inflammations produced by egg albumin and formalin and against granuloma formation by subcutaneous implantation of cotton pellets. Hamycin showed marked catabolic, thymolytic and adrenotrophic action. All these actions of hamycin are possibly mediated through a direct and prolonged stimulation of hypothalamus-hypophyseal region of central nervous system resulting in prolonged liberation of ACTH.

Authors acknowledge their thanks to Dr. M. J. Thirumalachar for his interest in the work.

1. Thirumalachar, M. J., Menon, S. K., Bhatt, V. V., Hindustan Antibiot. Bull., 1961, v3, 136.

2. Gokhale, B. B., Arch. Dermatol., 1963, v88, 558.

3. Shende, G. Y., Gogate, M. C., Padhye, A. A., Thirumalachar, M. J., Am. Rev. Resp. Dis., 1965, in press.

4. Dave, C. V., Hindustan Antibiot. Bull., 1964, v6, 181.

5. D'Amour, F. E., Blood, F. R., in Manual for Lab. Work in Mammalian Physiology, Univ. of Chicago Press, Ill., 1961, exp. 41.

6. Harris, J. M., Spencer, P. S. J., J. Pharm. Pharmacol., 1962, v14, 464.

7. Meier, R., Schuler, W., Desaulles, P., Experientia, 1950, v6, 469.

8. United States Pharmacopia XVII, Mack Publishing Co., Easton, Pa., 1965, p147.

9. Dave, C. V., Kaul, P. N., Hindustan Antibiot. Bull., 1964, v5, 119.

10. Kaul, P. N., ibid., 1964, v6, 112.

11. Sarett, L. H., Patchett, A. A., Steelman, S. L., in Progress in Drug Research, Jucker, E., Ed., Birkhäuser Verlag, Basel & Stuttgart, 1963, v5, 24.

12. Eskin, I. A., Mikhailova, N. V., Bull. Exp. Biol. Med., U.S.S.R., 1958, v8, 999.

13. Bennett, J. E., Williams, T. W., Piggott, W., Emmons, C. W., Proc. Soc. Exp. Biol. and Med., 1964, v117, 166.

14. Padhye, A. A., Thirumalachar, M. J., Hindustan Antibiot. Bull., 1963, v6, 41.

Received November 29, 1965. P.S.E.B.M., 1966, v122.

Changes in Lamb-Lung Lipids During Gestation.* (31049)

NOBUKAZU CHIDA, FORREST H. ADAMS, MASAHIKO NOZAKI, AND ANNE NORMAN
(Introduced by Allan Hemingway)
Department of Pediatrics, School of Medicine, University of California, Los Angeles

A considerable body of evidence has been presented recently to indicate that a substance with surface activity lines the internal surface of the lungs and that this substance helps to stabilize the alveoli during respiration(1). This surface active material, surfactant, is believed to be a lipoprotein with the activity residing in the phospholipid(2) fraction, particularly lecithin(3,4). Surfactant has been found in fetal lungs after 18 days in the mouse(5,6), 120 days in the lamb(7,8), and in human fetuses over 1,000 g in weight (9). The lungs of newborn infants dying of hyaline membrane disease usually show a reduction in surface activity(9,10) and a reduction in active phospholipid components (10).

The purpose of this report is to demonstrate both the quantitative and qualitative changes that occur in the lipid composition of saline extracts of lungs of normal fetal lambs during the last weeks of gestation. These will be related to the changes in surface activity.

Materials and methods. Fourteen fetal lambs at various gestational ages (based upon body weight) were delivered from ewes of varying genetic strains by cesarean section, taking special precautions to maintain the placental circulation intact(11). Lung specimens were obtained from each lamb by surgical biopsy and were kept frozen at —20°C until analyzed. Three grams of each lung specimen were finely minced in 50 ml of isotonic saline and then stirred for 20 minutes with a magnetic stirrer. The mixture was filtered through 2 layers of lipid-free gauze directly into a surface tension balance trough. After the surface tension measurements were made, the extract was lyophilized and used for lipid analysis. All lipid values were calculated as milligrams per 100 ml of lung saline extract.

Lipid analysis of lung saline extract. The solvents used were all American Chemical Society reagent grade and were redistilled prior to use. The filtrate of each lung saline extract was freeze-dried with a lyophilizer and was extracted in a nitrogen atmosphere, with chloroform:methanol (2:1 v/v). Total phospholipids were separated from the neutral lipids on a silicic acid column(12). The neutral lipids were eluted by chloroform and the phospholipids by methanol, and both were checked with thin layer chromatography. Lecithin, phosphatidyl ethanolamine and

* Supported by grants from USPHS.