bits impaired reproductive performance, infertility being complete when clover was fed both prior to and during gestation periods. Cause is unknown but failure of ovulation and implantation was observed, and evidence indicates that infertility may be unrelated to estrogenic content of the clover.

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Studies on Binding of Tetracyclines by Dog and Human Plasma.* (26130)

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Several investigators have studied the binding of tetracyclines by dog and human plasma. Binding of chlortetracycline in man has been reported by Sirota and Saltzman(1), Kunin *et al.*(2) and Kivman(3). Kunin *et al.*(2) have studied binding of demethylchlortetracycline in man, and Kohn *et al.*(4) in dog. The extent of protein binding of tetracycline by human plasma was investigated by Kunin *et al.*(2) and Kivman(3), and in dog plasma by Pindell *et al.*(5) and Kohn *et al.*(4). Not all the investigators agreed in their findings and different values were reported for one specific antibiotic and species.

The present paper deals with a comparative study of binding of demethylchlortetracycline with chlortetracycline and tetracycline by dog and human plasma. Extent of protein binding was determined by the dialysis equilibrium method, at drug concentrations approaching therapeutic levels and under physiological conditions of temperature and pH. The effect of temperature and pH on binding of tetracycline was also studied.

Method. The experiments were performed with a pool of dog plasma† (5 males and 6 females) and a pool of human plasma† (4 healthy males). Concentration of protein in plasma was determined by micro-Kjeldahl analyses. Distribution of plasma proteins was determined by free electrophoresis in the Spinco Model H electrophoresis apparatus.‡ Tetracyclines used were radioisotopically labeled as follows: 7-tritio-tetracycline hydrochloride, C^{14} demethylchlortetracycline hydrochloride and C^{14} chlortetracycline hydrochloride. Corresponding specific radioactivities were 13, 0.43 and 0.18 μc/mg. The purity of these tetracyclines, checked by various procedures including microbiological assay, spectrophotometric assay and paper chromatography, was 90% for demethylchlortetracycline hydrochloride, 95% for chlortetracycline hydrochloride and 100% for tetracycline hydrochloride. The technic followed for dialysis was developed by Roepke *et al.*(6). Small volumes (0.005-0.040 ml) of aqueous solutions of the tetracyclines were added to the plasma or to the buffer from a micro syringe-burette.§

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† Obtained from heparinized blood centrifuged with a layer of oil.

‡ Determined by Mr. M. C. Davies, Biochem. Research Dept.

§ Manufactured by California Laboratory Equipment Co.

TABLE I. Plasma Protein Distribution by Electrophoretic Separation.

Sample	Albumin	Globulins α_1	Globulins α_2	Globulins β	Globulins γ	Fibrinogen	Total protein, g/100 ml*
	%						
Pool of human plasma	57	4	10	11	12	6	8.6
" " dog "	48	5	7	13	18	9	6.1

* Determined by micro-Kjeldahl analyses.

A cellophane bag (7.6 cm long) of Visking Nojax 18/32 casing was used for dialyzing 1.5 ml of plasma. The bag contained a glass rod (7.6 cm long, 8 mm in diameter) which spread the plasma over a large area. The bag was sealed with a rubber band and placed in a ground glass stoppered test tube containing 10 ml of a solution of buffered saline (0.15M sodium chloride and 0.02M phosphate).‖ Unless otherwise stated the tube was rotated (at an angle approximately 12 degrees from horizontal axis of rotation) at 8 rpm for 4 hours inside an oven kept at 37°-38°C. Dialysis experiments lasting for 2, 4, 6 and 8 hours showed that equilibrium was attained between 2 to 4 hours. Final pH of the plasma and buffer measured at 37°C with a Cambridge pH meter was found to vary from 7.3 to 7.4. After equilibration, concentrations of tetracyclines in plasma and buffer were determined by a radiometric assay using liquid-scintillation counting as described by Takesue *et al.*(7). Degree of protein binding expressed as per cent bound of total drug in the plasma was calculated as follows:

$$\mu g \text{ unbound drug/ml plasma} = \mu g \text{ drug/ml buffer} \times \frac{\text{g water/ml plasma}}{\text{g water/ml buffer}}.$$

$$\% \text{ bound} = \frac{(\mu g \text{ total drug/ml plasma} - \mu g \text{ unbound drug/ml plasma})}{\mu g \text{ total drug/ml plasma}} 100.$$

Water content of plasma and buffer was determined by drying to constant weight. An average value of 0.948 was obtained for the ratio of water content of plasma to water content of buffer.

Since the same results were obtained for binding of tetracyclines with fresh and frozen plasma, the pools of plasma were preserved by storing them at –26°C.

‖ KH_2PO_4-NaOH buffer system.

Results. Separation of plasma proteins by electrophoresis (Table I) showed that the pools of plasma had a normal protein distribution.

Degree of binding of 3 tetracyclines by dog plasma proteins is summarized in Table II. The data show that over the range of concentrations covered (1-10 μg/ml) there was no effect of drug concentration on per cent of drug bound. Similar conclusions were derived from the data obtained with human plasma (Table III).

When pH was varied from 6.7 to 8.1 there was an appreciable increase in binding of tetracycline with increase in alkalinity (Fig. 1).

The effect of temperature on the equili-

TABLE II. Binding of Tetracyclines at Various Concentrations in Dog Plasma.

Drug	Drug conc. in plasma, μg/ml: Total	Unbound*	% bound
Tetracycline	.94	.61	35†
	.86	.56	35
	2.66	1.58	40
	2.42	1.61	33
	6.48	4.30	34†
	7.10	4.40	38
			36 ± 3‡
Demethylchlor-tetracycline	1.10	.56	49†
	1.09	.56	48
	2.80	1.41	50
	2.87	1.43	50
	8.69	4.38	50†
	8.13	4.58	44
			48 ± 2
Chlortetracycline	1.20	.38	68†
	1.20	.49	59
	2.79	1.33	52†
	3.56	1.49	58
	9.77	4.30	56†
	9.97	4.19	58
			58 ± 5

* $\frac{\text{g water/ml plasma}}{\text{g water/ml buffer}} \times \mu g \text{ drug/ml buffer} = \mu g$ unbound drug/ml plasma.

† Drug was added to buffer except in these determinations where it was added to plasma.

‡ Mean ± S.D.

brium was studied with tetracycline. When the dialysis equilibration was performed at 37-38°C, an average¶ value of 36% bound (33-40) was obtained. Slightly lower results, 30% bound¶ (27-33), were obtained at 2-5°C. The pH for this latter experiment was 7.5 at 2°C.

Discussion. The data reported here show that the tetracyclines (chlortetracycline, demethylchlortetracycline and tetracycline) are bound to a similar extent by dog and human plasma and are unlike the sulfonamides, which are bound to a greater extent in human than in dog plasma(8). Our study also indicates that the order of increasing binding tendency is tetracycline → demethylchlortetracycline → chlortetracycline. These findings are in agreement with the work of Kunin *et al.*(2) and Kohn *et al.*(4). As to absolute binding value for each specific antibiotic, our results for binding of tetracycline by dog plasma are significantly lower than those (69% bound) reported by Pindell *et al.*(5). While studying renal excretion of tetracycline in the dog, Sullivan and Fanelli, of these laboratories, found that the Krebs-Ringer-bicarbonate buffer(9) gave higher binding values than the phosphate-saline buffer used in our study.

TABLE III. Binding of Tetracyclines at Various Concentrations in Human Plasma.

Drug	Drug conc. in plasma, μg/ml Total	Unbound*	% bound
Tetracycline	2.16	1.34	38†
	1.84	1.31	29
	2.09	1.43	31
	6.10	4.07	33†
	6.02	4.42	26
			32 ± 4‡
Demethylchlortetracycline	1.13	.52	54†
	1.02	.47	54
	2.94	1.40	52†
	3.25	1.61	51
	3.05	1.58	48
	8.09	4.00	50†
	7.63	3.84	50
			51 ± 2
Chlortetracycline	1.37	.41	70†
	1.20	.48	60
	3.65	1.44	60†
	4.00	1.33	67
	4.12	1.51	63
	11.46	3.92	66†
	11.08	3.92	65
			64 ± 4

* $\frac{\text{g water/ml plasma}}{\text{g water/ml buffer}} \times \mu\text{g drug/ml buffer} = \mu\text{g}$ unbound drug/ml plasma.

† Drug was added to buffer except in these determinations where is was added to plasma.

‡ Mean ± S.D.

¶ Avg of 4 determinations. Ranges in parentheses.

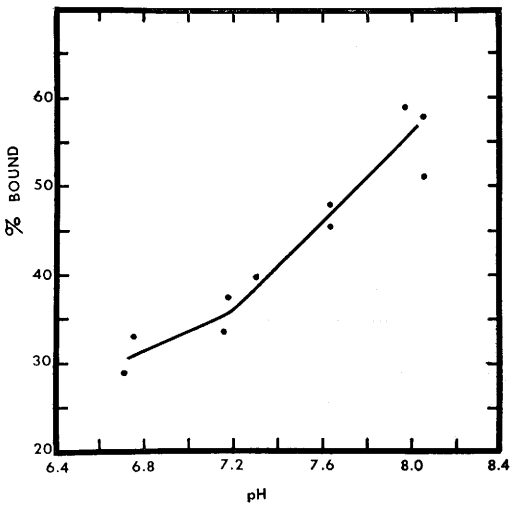

FIG. 1. Effect of pH on binding of tetracycline by dog plasma. Antibiotic concentration in plasma was 2-3 μg/ml.

Since the binding of drugs by plasma proteins is affected by concentration of drug in the plasma(10,11), protein concentration in the plasma(10), species,(11,12) pH,(10,13) temperature(10,14,15) and buffer, apparent disagreement in results from different investigators could be accounted for by variation in these conditions.

Summary. Dialysis equilibrium studies under physiological conditions of temperature and pH with tetracycline, demethylchlortetracycline and chlortetracycline have shown that degree of binding by dog and human plasma for drug concentrations ranging from 1-10 μg/ml was approximately twice as much for chlortetracycline (61%) as for tetracycline (34%), while demethylchlortetracycline was intermediate. These experiments also showed that each tetracycline was bound to a similar extent by dog and human plasma. A study with tetracycline showed that: a) approximately the same results were obtained when

the equilibrium was carried out at 37°C or at 4°C; and b) binding increased with increasing alkalinity over the pH range of 6.7 to 8.1.

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Zn^{65} Uptake by Rat Dorsolateral Prostate as Specific Bioassay for ICSH.* (26131)

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It has been demonstrated(1) that a measurement of the functional capacity of the rat dorsolateral prostate to concentrate Zn^{65} was a more sensitive indicator of ICSH (LH) activity than measurements of either dorsolateral or ventral prostate weight. The results of further studies to ascertain degree of sensitivity and specificity of Zn^{65} uptake as a bioassay for ICSH are reported in this paper.

Methods. Hypophysectomized (hypox) male Wistar rats (16-weeks of age, weighing 300 to 350 g) were purchased from Charles River Breeding Lab., Brookline, Mass. On the 5th day following surgery hormone injections were initiated as follows: 72 hypox rats received daily injections of 5, 20, 40, 100 and 200 μg of ICSH;† 52 hypox rats received daily injections of 200 and 500 μg of FSH,† Growth Hormone,† and Prolactin;† 12 hypox rats received daily injections of 20 and 40 μg of a human male castrate urine extract prepared and purified according to technics outlined by Albert(2). All hormone preparations were injected intramuscularly in 0.2 ml physiological saline, once daily for 6 days. Eighteen hypox rats injected with 0.2 ml physiological saline daily served as controls. On the 6th day of hormone administration all rats received intracardiac injections of Zn^{65} (0.04 μc/g) as outlined previously(3). Twenty-four hours later the rats were sacrificed, the dorsolateral prostates dissected(4), and their radioactive content determined by technics described(3). All rats used in this experiment were inspected grossly for completeness of hypophysectomy. None showed any pituitary remnants. During this investigation the rats were housed under constant

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