a more active form, by rat tissues, is required, rate of utilization would be relatively slower and a 24-hour test might well be too short a period for quantitative comparison.

It is interesting to note that, following a single injection, whale and pig growth hormones had the same duration of activity and magnitude of response as did human, monkey, and beef preparations in the time intervals studied. This could be a further indication that the difference between the 2 assays is a function of time dependent upon modification of the growth hormone molecule and/or slower rate of utilization of whale and pig material.

The fact that the 2 assays for growth-promoting activity give quantitatively different results suggests that the short-term assay is of value for qualitative measurement and that quantitative comparisons require a longer test period.

Summary. Growth hormone isolated from 6 different species was compared for its ability to increase width of epiphyseal plate and to stimulate radio-sulfate incorporation by costal cartilage. Human, beef, monkey, and sheep preparations manifested almost identical effects in the 2 assays. Whale and pig hormones appeared to elicit greater effect in the tibia test. Whale growth hormone was observed to have greater duration of activity to stimulate radio-sulfate incorporation than other preparations studied. Short-term radio-sulfate assay appears to be of value for qualitative detection of growth-promoting activity.

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Determination of "Heparinoid" Substances in Urine with a Dye-Binding Technic.* (26613)

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Although crystalline heparin of known chemical composition and with reproducible physiological properties is available for clinical and laboratory study, the basis of its action in blood clotting and other metabolic activities has not been completely elucidated. Complicated chemical methods have revealed very small amounts of heparin in the circulating blood, too minute, however, for quantitative determination.

Early investigators were not in agreement as to the fate of heparin, whether endogenous or administered. Howell and McDonald(1) were first to report the presence of heparin in urine of dogs following injection. Copley and Schnedorf(2) using a relatively simple dye-precipitation technic, found that from 9.9-35.6% of injected heparin was excreted in urine of dogs within 110 minutes. There was no activity before administration. Jaques(3), using a metachromatic technic, and Astrup(4), with an anticoagulant procedure, reported daily excretion in urine of man of amounts up to 0.7 mg heparin without prior injection. When injected, excretions of 2-15%(3) and 1.6-12%(4) were obtained.

The excreted material is not unchanged heparin, as seen in the disparity between metachromatic activity and the lesser clot-

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inhibiting ability of the isolated substance (3-7). *In vivo,* these and other related substances have been characterized as "heparinoid"(8).

The method was developed from the procedures of Copley(2,9), modified and made quantitatively more accurate and reproducible. Following centrifugation of a mixture of heparin and toluidine blue, an inverse linear relationship was found to exist between concentration of heparin and optical density of the unreacted dye supernatant.

Indirect determination of heparin or other dye-binding substances ("heparinoids") by separation in the insoluble complex with toluidine blue appeared to be a logical approach.

Methods and materials. One milliliter of toluidine blue‡ solution, 0.0125%, in 0.067 molar phosphate buffer, pH 7.2, is added to 0.5 ml of centrifuged test urine in a microcuvette (10×75 mm). The solution is mixed by tapping and allowed to stand for 15 minutes at room temperature. The dye and "heparinoids" react to form an insoluble purple complex which settles out during 5 minutes of centrifugation. The supernatant is not decanted and its per cent transmittance or optical density is read in a spectrophotometer at 700 mμ against a blank of urine and buffer. Units of reacting substance are interpolated from a standard calibration graph and are reported as total units of "heparinoids" excreted per hour. The standard graph is prepared by reacting 1.0 ml toluidine blue solution with 0.5 ml volumes of phosphate buffer containing 1 to 5 units of heparin§ respectively. The standard graph should be checked for each new preparation of diluted dye solution, since small fluctuations occasionally occur. The heparin dilutions are stable for several months, refrigerated.

‡ Supplied by Abbott Laboratories, North Chicago, Ill. A stock solution of 0.200% powdered dye in the phosphate buffer was prepared.

§ Supplied by Lederle Laboratories, Pearl River, N. Y.;
100 mg/ml
100 units/mg

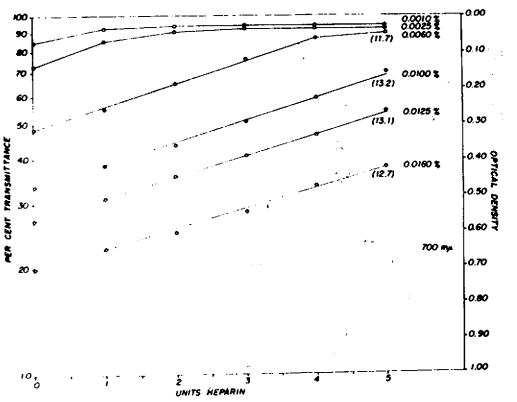

FIG. 1. Relationship between per cent transmittance (optical density) and concentration of heparin at 700 mμ for several concentrations of toluidine blue. The gamma of dye decolorized by one unit heparin is indicated in parenthesis. The value at 0.0060% was determined for the major linear portion of graph.

The subjects tested were normal individuals who were in no way restricted during the test. A 2-hour urine specimen was collected just prior to administration of 25 mg (2500 units) of heparin. Injection was made slowly, admixing with blood, with a tuberculin syringe. The urine voided during the 2-hour period following injection was collected and both specimens were analyzed. The increase of dye-binding substances in the second specimen over that in the first was calculated and reported as per cent of injected heparin.

Results. Utilization of 700 mμ and 0.0125% toluidine blue (Fig. 1) best meets the requirements of sensitivity and linearity of response over the range encountered for the test. Identical graphs were obtained for freshly prepared reaction mixtures at pH 5.5, 7.2, and 8.3, whether in distilled water or phosphate buffer at 0.067 or 0.133 molar. The dye at pH 5.5 and 7.2 is stable, but at pH 8.3 there is considerable fading of color within a week, necessitating constant restandardization. The reaction of most freshly voided urines is slightly acidic and after addition of the pH 7.2 buffered dye solution the pH of the mixture falls at about 6.7 ± 0.5. Under these conditions duplicate analyses are in excellent agreement and reproducible results are obtained with urines

TABLE I. Enhancing Effect of Urine on Reaction between Heparin and Toluidine Blue.

			"Heparinoid" units†			
			Before dialysis		After dialysis	
	Urine, ml	Heparin, ml*	Theoretical	Analytical	Theoretical	Analytical
A.	.25	1.75	4.41	4.73	4.33	4.55
	.50	1.50	3.81	4.35	3.86	4.00
	1.00	1.00	2.62	3.17	2.71	2.82
	1.50	.50	1.43	1.80	1.57	1.62
	1.75	.25	.84	.90	1.00	1.04
	2.00	.00	—	.24	—	.40
B.	.25	1.75	4.56	4.94	4.56	4.58
	.50	1.50	4.11	4.63	4.10	4.10
	1.00	1.00	3.22	3.75	3.20	3.27
	1.50	.50	2.33	2.60	2.30	2.31
	1.75	.25	1.89	1.95	1.83	1.85
	2.00	.00	—	1.44	—	1.40

* 10 units/ml.

† Per ½ ml test mixture.

stored at refrigerator temperature for periods up to 3 weeks.

The amount of dye (gamma) reacting with one unit of heparin at different concentrations of toluidine blue is indicated in Fig. 1. These were determined by calculating decrease in optical density per unit heparin, and units of optical density per gamma of toluidine blue. One unit of heparin was found to react with (decolorize) 13 γ of dye. This agrees with the value of 15 γ reported by Copley and Whitney(10), and calculated by the present authors from the graph of MacIntosh(5).

The competitive action of proteins for heparin(11) in reaction with toluidine blue was studied because of the occasional presence of trace amounts of albumin in urine of some patients. The data obtained showed there is no interference with urinary "heparinoid" (or pure heparin) at serum protein concentrations up to 1.0% for "heparinoid" levels as high as 3.5 units per 0.5 ml test specimen. When present in larger amounts, the protein does prevent the heparin from reacting with the dye, and false low results are obtained. The toluidine blue test is thus of limited value for specimens with a proteinuria greater than +2, and the specimen must be diluted before analysis.

There is an enhancing substance present in urine, which is responsible for a marked though variable increment in recovery experiments. The results of 44 such experiments show the enhancement to vary from 9-43%. This effect is present even in urines containing no measurable dye-binding substance, and has been noted by Jaques(3). After prolonged dialysis of urine, mixtures with heparin yield analytical results in agreement with theoretical values. Table I presents recovery data before and after dialysis for 2 urines of different "heparinoid" content. That the enhancing material has an effect only on added heparin in mixtures with urine, and not on excreted "heparinoids" is shown by the fact that results for dye-binding substance are the same before and after dialysis.

The enhancing factor is not related to urinary pigment since dark and light colored urines of the same content of dye-binding substance were equally effective. When aliquots of several urine specimens are mixed, there is no enhancing effect noted. Thoretical and analytical results are in agreement.

In Table II are found mean hourly excretions of dye-binding substances in the urine of normal subjects. Specimens were collected for varying intervals of time (2-10

TABLE II. Excretion of "Heparinoids" in Urine of Normal Subjects.

Subject	Age	No. of subjects	No. of specimens	"Heparinoid" units/hr
Males	20-53 yr	24	139	29 ± 11*
Females	18-52 "	31	95	16 ± 13
Children	2-12 "	25	40	80 ± 31
Infants	1- 8 days	18	18	16 ± 14

* Mean ± S.D.

hours) during all parts of the day. There was no significant variation during any part of the day. When more than one specimen from an individual was tested, the values were averaged before being included in determination of the mean. One premature infant (male) was studied weekly for 6 weeks. His values were the same as that of the full-term infants. The difference in values for men, women, and children (boys and girls) is significant. For the men and women, $t = 3.8$. Several specimens daily, representing from 16-24 hours, were collected from 2 adults for extended periods and analyzed. Daily averages were calculated, their mean determined and reported as units of "heparinoid". For the male, the mean of 13 days (42 specimens) was 34 units ± S.D. 10 units "heparnoid" per hour; for the female, the mean of 14 days (29 specimens) was 12 units ± S.D. 11 units "heparinoid" per hour. These values agree with the mean for their groups.

Following an intravenous test dose of 25 mg heparin, 14.5% ± S.D. 4.1% was excreted in a group of 27 normal men and women ranging in age from 16-72 years. There was no significant difference between the sexes in this respect.

The results of a study of 31 women making weekly visits to the prenatal clinic during the second half of their pregnancy are presented in Table III. A substantial increase in excretion of dye-binding substance is noted as early as the 6th month and probably exists earlier. Peak levels are reached during the middle of the third trimester. These increments over normal approximate those following the test dose. Twelve women were followed daily during their 7 days of post-partum care and the mean of 55 specimens indicates a return to normal levels.

Discussion. Analyses of urine specimens before injection of the standard dose revealed the presence of measurable amounts of reacting material. Substantial quantities of dye-binding substance were found in the urine of children (50-120 units "heparinoid" per hour). In infants small amounts approximating those found in women are excreted. The urine of women (0-30 units "heparinoid" per hour) contains less than that of men (20-40 units "heparinoid" per hour). There is no variation during the menstrual cycle. A similar significant difference in the urines of men, women and children has been reported by other investigators(12-14) using 2 entirely different analytical procedures for measurement of acid mucopolysaccharides. These compounds reacted with toluidine blue and when chromatographed on paper were identified chiefly as chondroitin sulfate.

TABLE III. Excretion of "Heparinoids" during Pregnancy.

Wk before parturition	Subjects tested	"Heparinoid" units/hr, mean
9–14	19	91
8	4	85
7	10	71
6	7	119
5	12	125
4	15	113
3	21	124
2	24	114
1	21	137
Daily, 1 wk after delivery	12	65

The great increase during pregnancy presumably reflects the overall change in body metabolism. Peak levels are reached in the middle of the third trimester, followed by a decrease in the first post-partum week. In a study of post-operative patients a pronounced increase in excretion of dye-binding substance occurred on the first or second day followed by a gradual and sometimes erratic subsidence to pre-operative levels.

Conclusions. 1. A quantitative method based on the reaction of toluidine blue with heparin and "heparinoids" is described. It reveals a range of 9-24% output in the urine of injected heparin in normal individuals following a standard test dose of 25 mg of heparin. 2. The presence of dye-binding substances in varying amounts in the urine of infants, children, men, women, and during pregnancy, without an injection of heparin, suggests the existence of a "heparinoid" metabolism.

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A Serological Reaction Associated with Sarcoidosis.* (26614)

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At present the diagnosis of sarcoidosis is based on clinical grounds, supported by either a compatible histological pattern or a positive Kveim test(1). Since many other agents are known to produce the so-called "hard tubercle"(2) and since a potent and reliable Kveim antigen is difficult to obtain, final diagnosis is often accomplished by exclusion.

When potent Kveim antigen is available positive reactions are obtained in about 75% of cases(3). If another positive test possessing a fair degree of consistency can be found, the uncertainties resulting from diagnosis by exclusion may be somewhat reduced. Since the reported distribution of non-photochromogenic anonymous mycobacteria resembles somewhat the distribution of sarcoidosis as described in Veterans Administration studies, it seemed worth while to study sera of patients with sarcoidosis against antigens of various types of anonymous mycobacteria. If antibodies should be found they might also suggest clues as to the etiology of sarcoidosis.

The following studies were undertaken to see if serum of patients with sarcoidosis possessed antibodies detectable by diffusion against any of a number of mycobacterial and fungal antigens. Diffusion in agar was chosen as a method since Parlett and Youmans(4) have found the technic useful in mycobacterial study, while Heiner(5) has used diffusion in the study of histoplasmosis, and experience in this laboratory indicates its usefulness in other fungus infections.

Materials and methods. Sera from 32 patients with sarcodosis as determined by usual clinical criteria supported by Kveim test, biopsy or both were subjected to double diffusion in agar gel. These sera were distributed geographically as follows: Virginia 8, Wisconsin 5, Oklahoma 5, Louisiana 7, and Texas 7.

Diffusion was carried out in Petri dishes, using wells and distances as described by Crowle for slide technic(6). A 1% solution of agar was poured in the dish and allowed to harden. A second pour of 1½% ordinary bacto-agar in physiological saline was then layered on the first pour, and as it first began to harden a tooled die was placed on the surface, forming wells of a constant size and a fixed distance.

The central well was filled with undiluted serum and the peripheral wells with various antigens. In a few instances when the first

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