fect at the stem cell level would be expected to result in production of normal erythrocytes. Thus, there is no evidence to indicate that erythropoietin is part of an abnormal mechanism.

Summary. Erythrocytes produced in rats under stimulus of human urinary erythropoietin have a normal survival time in circulation. This and other evidence suggest that erythropoietin administration results in production of normal erythrocytes. This evidence is compatible with the concept that erythropoietin is part of the normal mechanism controlling erythropoiesis.

1. Gordon, A. S., *Physiol. Rev.,* 1959, v39, 1.

2. Gordon, A. S., Winkert, J. W., Dornfest, B. S., LoBue, J., Crusco, A., *The Kinetics of Cellular Proliferation,* Grune and Stratton, 1959, N. Y. and London.

3. Berlin, N. I., Waldmann, T. A., Weissman, S. M., *Physiol. Rev.,* 1959, v39, 577.

4. Garcia, J. F., Van Dyke, D. C., *J. Appl. Physiol.,* 1959, v14, 233.

5. White, W. F., Egan, R., Schlueter, R. J., Wever, G. F., Goldwasser, E., ACRH-8, 1957, 130.

6. Berlin, N. I., Lawrence, J. H., Lee, H. C., *J. Lab. Clin. Med.,* 1954, v44, 860.

7. Fryers, G. R., Berlin, N. I., *Am. J. Physiol.,* 1952, v171, 465.

8. Berlin, N. I., Van Dyke, D. C., Lotz, C., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 287.

9. Van Dyke, D. C., Asling, C. W., Berlin, N. I., Harrison, R. G., *ibid.,* 1955, v88, 488.

10. Belcher, E. H., Harriss, E. B., *J. Physiol.,* 1959, v146, 217.

11. Alpen, E. L., Cranmore, D., *The Kinetics of Cellular Proliferation.* Grune and Stratton, 1959, N. Y. and London.

12. Alpen, E. L., Lajtha, L. G., Van Dyke, D. C., *Nature,* 1959, v184, 1228.

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Direct Measurement of Radioactive Sulfate Uptake by Rat Mast Cells.* (25913)

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The *in vivo* uptake of $S^{35}O_4$ by an organic component of mast cells has been demonstrated using autoradiography. By means of intensity grading(1) or grain counting(2) an estimate of rate of uptake and loss of radioactivity was achieved. The grading method is at best semi-quantitative, while the grain counting technic is cumbersome. Therefore we searched for a quantitative method of reasonable simplicity. Millipore filters have proved useful in several types of cytologic study. Collection of free cells on these filters has been employed in clinical cancer cytology (3,4), in *in vitro* studies of cell function(5) and in tissue culture(6). This study was undertaken to extend this method of cell collection to permit direct measurement of incorporation of radioactively labeled substances into cells. The cells chosen for the investigation were free peritoneal mast cells of the rat. Radioacitve sulfate was used as the tracer compound, since sulfate is known to be incorporated into mast cell heparin(7).

Materials and methods. Isotope labeled sulfate obtained as $H_2S^{35}O_4$ in dilute HCl was titrated to neutrality with NaOH and diluted to the desired concentration with .8% aqueous NaCl. This solution was administered intravenously in the tail of an etherized rat. Sprague-Dawley male rats weighing 280-390 g were used in most experiments. Mast cells were obtained from rat peritoneal cavities by the procedure previously described(8). Separation of mast cells from other peritoneal cells was achieved by centrifugation through

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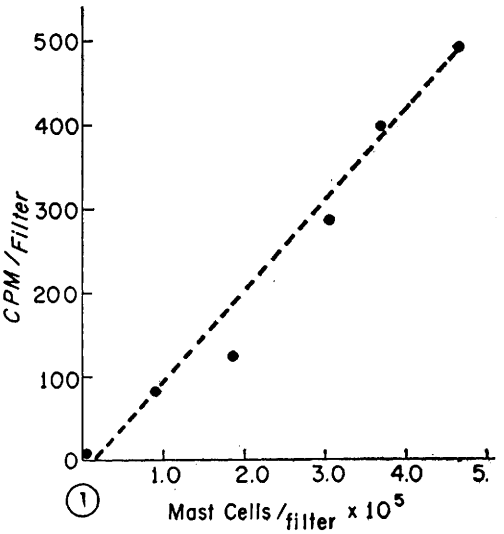
concentrated sucrose solution(9). Samples of the cells in the initial peritoneal washings as well as the separated cell fractions were collected on Millipore filters, Type AA, 1 inch diameter, using the standard chimney assembly (purchased from Millipore Filter Corp., Watertown, Mass). The cells were washed with Tyrode's solution and the filters allowed to dry on the absorbent pads supplied with the filters. A glass slide placed over the filter prevented curling during drying. The Millipore filter on the pad was then placed directly in a 1 inch inner diameter planchette and the radioactivity measured. A GM tube with a "micromil" window (Nuclear, Chicago) was used. Counting rates were recorded in counts per minute (CPM) and were corrected for background. No corrections for isotope decay were necessary since all measurements within a single experiment were made on the same day. Counting rates were low enough to obviate a correction for resolving time. A small number of autoradiographs were prepared by the coating emulsion technic. Following 4% formaldehyde fixation, the filters were dried between 2 glass slides, attached to a slide with nitrocellulose and coated with NTB-3 emulsion (generously supplied by Eastman Kodak Co., Rochester, N. Y.). The emulsion was then exposed for 14 days at 4°C and developed in Kodak Dektol. The filters were stained with .1% aqueous safranin O, dehydrated through alcohol, cleared in xylene and mounted in permount.

Results. Examination of toluidine blue stained Millipore filter preparations revealed the mast cells and other cells to be distributed in a single layer on the filter. The conditions of counting therefore were those of a very thin layer, uniformity of the layer depending on cell thickness. In order to test the feasibility of direct counting on the filter, counts per minute were measured for varying numbers of $S^{35}O_4$ containing mast cells from the same animal. The data from this experiment are presented in Fig. 1. The coefficient of correlation of numbers of cells with CPM was + .98, indicating linearity within the range of the numbers of cells used.

A study of the relationship of administered dose of $S^{35}O_4$ to radioactivity incorporated into the mast cells provided a further test of the method. A linear relation was obtained (Fig. 2).

Since the first 2 experiments were conducted with complete peritoneal cell washings it was



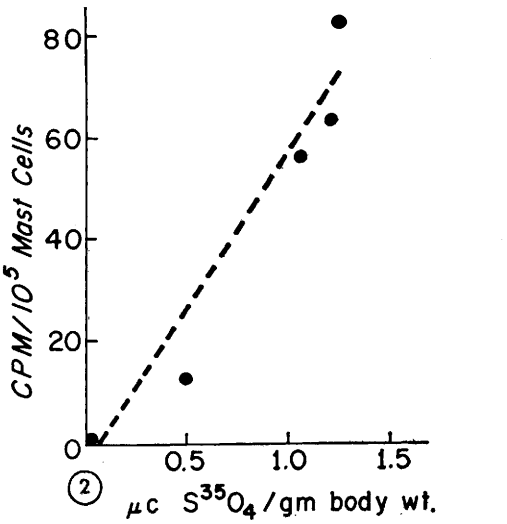


FIG. 1. Relationship between CPM/Filter and No. of mast cells/filter. Initial peritoneal washing from one animal was diluted to give varying numbers of $S^{35}O_4$ labeled mast cells. Two filters were prepared at each dilution and counting was carried out for 3 min.

FIG. 2. Effect of dose of $S^{35}O_4$ administered on CPM/10^5 mast cells 24 hr after I.V. administration. Each point represents avg of counts on 2 filters prepared from one animal. Counting was carried out for 3 min.

of interest to know whether or not all the radioactive label was present in the mast cells and further to examine the rate of incorporation of $S^{35}O_4$ into mast cells. Ten rats were given 1.25 μc/g body wt. of $S^{35}O_4$ intravenously, and at each of the times, 2, 4, 16, 48 and 168 hours following administration 2 animals were sacrificed. Radioactivity of the whole cell population, the mast cell fraction and the interface mixed cell fraction was measured on 2 samples from each of the animals. Mast cells comprised, by number, 5 $\pm$ 2% of the initial peritoneal washings, 94 $\pm$ 4% of the mast cell fraction (MC Fraction) and 1 $\pm$ 0.3% of the other peritoneal cell fraction (OPC Fraction). Since neither the MC fraction nor the OPC fraction constituted a uniform cell population, the values for CPM per 10^5 mast cells $\frac{\text{CPM}}{10^5\ \text{MC}}$ and CPM per 10^5 other peritoneal cells $\frac{\text{CPM}}{10^5\ \text{OPC}}$ were derived from the solution of 2 simultaneous equations:

$$1.\ \frac{\text{CPM}}{10^5\ \text{OPC}} \cdot \frac{\text{OPC} \times 10^5}{\text{Filter}} + \frac{\text{CPM}}{10^5\ \text{MC}} \cdot \frac{\text{MC} \times 10^5}{\text{Filter}} = \frac{\text{CPM}_{\text{MC Fraction}}}{\text{Filter}}$$

$$2.\ \frac{\text{CPM}}{10^5\ \text{OPC}} \cdot \frac{\text{OPC} \times 10^5}{\text{Filter}} + \frac{\text{CPM}}{10^5\ \text{MC}} \cdot \frac{\text{MC} \times 10^5}{\text{Filter}} = \frac{\text{CPM}_{\text{OPC Fraction}}}{\text{Filter}}$$

Data for radioactivity of the mast cells and the other peritoneal cells are presented graphically in Fig. 3. By 24 hours radioactivity of the mast cells accounted for 89% of the radioactivity in the total peritoneal cell population.

The internal consistency of the values obtained for the $\frac{\text{CPM}}{10^5}$ MC Fraction and $\frac{\text{CPM}}{10^5}$ OPC Fraction was established by multiplying these values by the respective numbers of cells in the initial washings. The sums of these two products, representing the total $\frac{\text{CPM}}{\text{Filter}}$ expected, were compared to the values obtained from direct measurements of filters prepared from the initial washings. Close agreement was observed as seen in Table I.

TABLE I. Comparison of Expected and Obtained Radioactivity of the Initial Washings.

Time, hr	CPM* Expected	Found
2	113	125
4	106	107
16	100	114
48	182	168
168	199	177

* Avg values from 2 animals with 2 filters counted on each animal.

An analysis of the errors of measurement was made. When CPM for 27 pairs of duplicate filters were compared, the mean deviation from the means of the pairs was 9.1% with a standard deviation of $\pm$ 7%. This variation is accounted for by errors in pipette measurements of aliquots delivered to the filters and the probable lack of uniform distribution of cells in the solution. This lat-

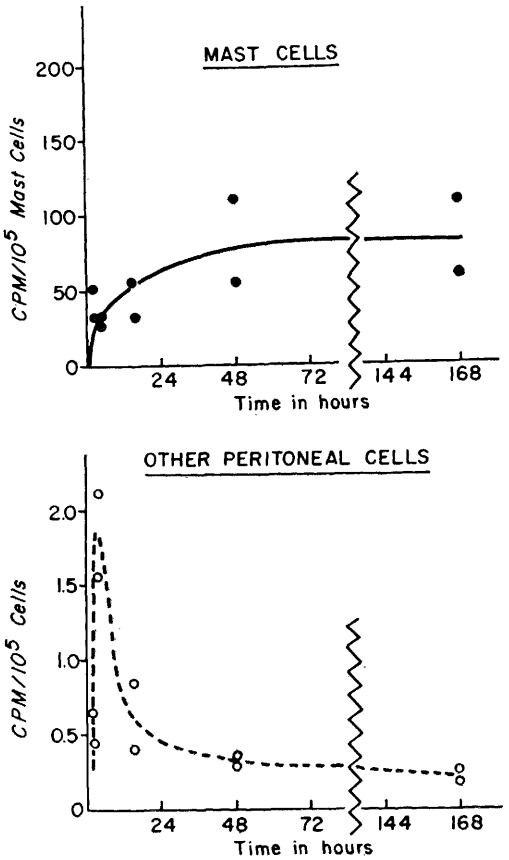


FIG. 3. Incorporation of $S^{35}O_4$ in peritoneal cells. Each point represents the avg of counts on 2 filters prepared from one animal. Time required to accumulate 1000 counts was measured.

ter factor is of particular significance in the viscous sucrose solutions. The larger variation obtained when results from 2 animals were compared (mean deviation from the mean of a pair was 20% with a standard deviation of ± 10% for 10 pairs) was presumably a function of biological variation and variations in cell counts. This latter error is remediable by counting a greater number of cells.

Discussion. Recent evidence has confirmed the fact that heparin is the sulfated mucopolysaccharide which confers on mast cells their characteristic metachromasia(7,10,11). *In vitro* experiments with a serially propagated mouse mast cell tumor have elucidated in part the mechanism of incorporation of sulfate into mast cell heparin(12). Though it is probable that most of the sulfate incorporated into mast cells in our studies is located in the heparin, definite proof of the chemical site of incorporation requires extraction and identification of the $S^{35}O_4$ containing substance(s).

Our experiments indicate that peritoneal mast cells do incorporate intravenously administered sulfate and that the loss of the sulfate is sufficiently low to be indiscernible within a period of 7 days. Guidotti(2) employing autoradiography in a study of SO_4 metabolism by tissue mast cells, found a 50% loss of activity on the basis of track counts in adult rat abdominal skin mast cells in the period between 48 and 144 hours after a single I.V. dose of $S^{35}O_4$. Our data are too scant to assert categorically a significant difference from Guidotti's results. The apparent discrepancy between his results and those of Jorpes *et al.*(1) is similarly in question. Further quantitative data with careful control of such parameters as site of origin of mast cells, and age and sex of rats employed are required to resolve the problem.

The Millipore filter method for direct determination of mast cell radioactivity should permit study of rate of incorporation and turnover of sulfate in the cells under various conditions known or postulated to affect mast cells such as antigen-antibody reactions, 48/80 treatment, hormonal excess or deficiency, and aging. The method provides a measure of mast cell activity quite different from changes in number, morphology and amine content previously relied on for information in elucidating the function of mast cells.

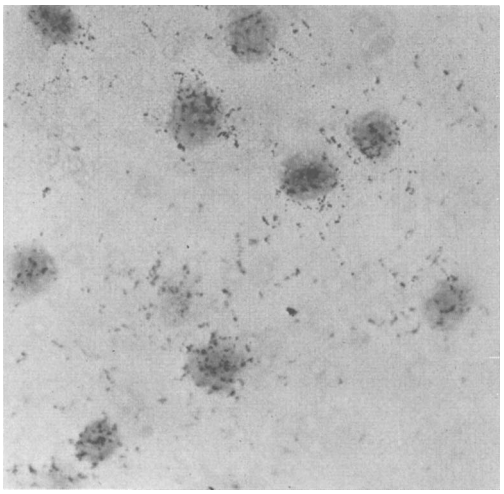


FIG. 4. Autoradiograph at 24 hr of peritoneal cells, prepared by coating emulsion technic. 14 days exposure at 4°C. Stained with .1% Safranin O. The large stained cells are the mast cells; the other cells appear only as faint ghosts.

The significance of the presence of $S^{35}O_4$ in the peritoneal cells other than mast cells 4 hours after administration of the sulfate is not known. Since this fraction is diverse, including neutrophils, eosinophils and lymphocytes, it may be that only one of these cell types is responsible for the radioactivity of this fraction. Careful autoradiography should reveal any non-uniform distribution of the $S^{35}O_4$ in these cells.

The method of direct counting of a monolayer of radioactively labeled cells on Millipore filters in conjunction with cell separation technics has proved applicable to study of sulfate incorporation by mast cells. The method is potentially useful for studies of other cells found in suspension normally or separated from tissues or tissue culture outgrowths with agents such as trypsin and ethylenediaminetetraacetic acid. The application of the Millipore method of collecting mast cells has been reported in an examination of the mechanism of degranulation(13).

Summary. 1) Radioactivity of $S^{35}O_4$ labeled cells can be measured directly with cells

collected on Millipore filters. 2) Following a single intravenous injection of $S^{35}O_4$ rat peritoneal mast cells take up the radioactivity; the maximum is reached by 48 hours. No loss of radioactivity is evident in the following 5 days. Radioactivity in other free cells of peritoneal fluid attains a maximum at 4 hours and then falls rapidly to low levels.

1. Jorpes, E., Odeblad, E., Bostrom, H., *Acta Haematol.*, 1953, v9, 273.
2. Guidotti, G., *Exp. Cell Res.*, 1957, v12, 659.
3. Seal, S. H., *Cancer*, 1956, v9, 866.
4. Del Vecchio, P. R., DeWitt, S. H., Borelli, J. I., Ward, J. B., Wood, T. A., Jr., Malmgren, R. A., *J. Nat. Canc. Inst.*, 1959, v22, 427.
5. Oxender, D. L., Christensen, H. N., *J. Biol. Chem.*, 1959, v234, 2321.
6. Grobstein, C., *Exp. Cell Res.*, 1956, v10, 424.
7. Korn, E., *J. Biol. Chem.*, 1959, v234, 1325.
8. Lagunoff, D., Benditt, E. P., *Am. J. Physiol.*, 1959, v196, 993.
9. Benditt, E. P., Arase, M., *J. Exp. Med.*, 1959, v110, 451.
10. Benditt, E. P., *Ann. N. Y. Acad. Sci.*, 1958, v73, 204.
11. Schiller, S., Dorfman, A., *Biochim. and Biophys. Acta*, 1959, v31, 278.
12. Korn, E., *J. Biol. Chem.*, 1959, v234, 1649.
13. Lagunoff, D., Benditt, E. P., *J. Exp. Med.*, 1960, v112, in press.

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Local Shwartzman Reaction in Rabbit Oral Mucosa with Endotoxin from Oral Bacteria.* (25914)

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Although endotoxins from many bacteria have been isolated and tested for toxicity, little work has been devoted to endotoxins from oral microorganisms. Recently, endotoxins from oral bacteria, isolated by tryptic digestion of cells and also by the phenol-water extraction method, were shown to possess many chemical properties and biological activities characteristic of classical lipopolysaccharide endotoxins(1,2). Primary toxicity to rabbit skin was demonstrated, and the localized Shwartzman reaction of hemorrhagic necrosis was produced by administering these endotoxic preparations first locally into skin, and then intravenously. The local Shwartzman effect has been achieved with many endotoxins in different tissues(3,4) yet no successful attempts seem to have been made to produce this reaction in tissues of the oral cavity. Because of the possibility that bacterial endotoxins may have etiologic significance in oral soft tissue diseases either through primary local toxicity or through Shwartzman-like effects, one of the recently isolated endotoxins was tested in rabbit oral mucous membrane.

Materials and methods. New Zealand white rabbits weighing 1.7 to 2.5 kg were anesthetized by intravenous injection of 40 mg per kg of pentobarbital and access to the posterior oral region was obtained by means of a rodent mouth prop previously described (5). With a modified 30 gauge needle, 50-100 μg (0.2 ml) of an endotoxin prepared by the phenol-water method from *Veillonella* organisms(1) was injected into an area which included the gingiva and palatal mucosa adjacent to the last 2 upper molar teeth. The corresponding site of the contralateral side was given a control injection either of 100 μg (0.2 ml) of soluble products from a human oral strain of viridans streptococcus or of 0.2 ml of 0.85% sodium chloride (saline). The streptococcal products contained proteins(6), carbohydrates (Molisch), and chloroform-soluble lipids, derived also by the phenol-water extraction procedure. Comparable dos-

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