An evaluation of radiosulfate as a granulocyte label in the dog

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The uptake of radiosulfate by dog bone marrow and blood cells was investigated both in vitro and after intravenous administration. The findings confirm previous observations that radiosulfate binds primarily to myeloid precursors and megakaryocytes and extends these observations to give a semiquantitative estimate of the relative degree of labeling of the various cell types. After intravenous administration (in vivo labeling), radiosulfate was bound initially to myelocytes (eosinophilic and neutrophilic) as judged from autoradiographs. The subsequent appearance of labeled granulocytes in the blood gave a curve very similar to that reported for radiophosphate and tritiated thymidine. The radiosulfate disappeared slowly from marrow myelocytes; the median grain count per labeled myelocyte decreased by half approximately every 24 hours. This finding is in reasonable agreement with published estimates of 11 to 20 hours for dog myelocyte generation time. The slow decrease in marrow myelocyte radioactivity with time does not appear to be compatible with the classic scheme of myelocyte division in which all myelocyte progeny mature and enter the next compartment after only one cell division. Rather, the findings appear to be more compatible with either several serial generations of myelocytes which divide according to the classic scheme or with a single generation of myelocytes of the type in which one half of the progeny mature and the other half remain myelocytes and can divide again.

The uptake of radioactive sulfate in the bone marrow,[1, 2] primarily in young myeloid forms and megakaryocytes,[3-6] and the subsequent appearance of labeled platelets[5] and leukocytes[6, 7] in the blood have been described in several species. These studies stimulated us to investigate further the use of radiosulfate as a granulocyte label. It was postulated that radiosulfate uptake by marrow granulocyte precursors might be restricted initially to one or two stages, such as myelocytes and promyelocytes.[4] If such were the case, the subsequent change in pro-

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portion of cells labeled and grains per cell, as determined by autoradiographic means, might permit inferences about myelocyte division. Accordingly, dog blood and bone marrow were incubated in vitro with radiosulfate, and the dogs were given radiosulfate intravenously. The relative quantity of radiosulfate uptake by blood and bone marrow cells was recorded. In addition, after in vivo radiosulfate administration, the changes in marrow myelocyte label and blood granulocyte label with time were followed. The results of these studies are described here.

Materials and methods

Animals. Mongrel dogs weighing 11 to 15 kilograms were used in these studies. Control leukocyte and differential counts were obtained before commencing all studies, and control bone marrow differential counts were made where appropriate. Either pentobarbital,* 30 mg. per kilogram, or thiopental sodium,† 15 mg. per kilogram, was used to anesthetize the dogs during bone marrow aspirations or prolonged infusions. Carrier free sodium sulfate S^{35}, 524 to 756 μc per milliliter, in sterile solution was obtained from Abbott Laboratories, Oak Ridge, Tenn.

Labeling procedures and radioactivity measurement. In vivo labeling was accomplished by infusing radiosulfate, diluted approximately tenfold with normal saline, into a foreleg vein. Infusion required from 5 to 20 minutes. Seven dogs were given 2 to 15 mc. (.176 to 1.04 mc. per kilogram) of radiosulfate for study of the blood granulocyte radioactivity curve. At daily intervals thereafter, 20 ml. samples of blood were drawn from an internal jugular vein and placed into 40 ml. of 3 per cent dextran (molecular weight approximately 225,000) in 0.9 per cent saline. One hundred units of heparin was used as an anticoagulant. After mixing, the samples were allowed to sit for 30 minutes to permit sedimentation of the red blood cells. The leukocyte rich supernatant was removed and granulocytes were separated from erythrocytes and platelets by the method of Raab and associates.[8] Granulocyte radioactivity was measured in a plastic scintillation system and expressed as counts per minute per milligram of granulocyte nitrogen.[9]

Two additional dogs were given 10 and 15 mc. (0.85 and 1.04 mc. per kilogram) of radiosulfate, respectively, to produce heavy labeling of the bone marrow for autoradiographic study. Bone marrow samples were obtained from the sternum and a rib at 7, 24, 48, 72, and 96 hours after labeling. Marrow particle smears were made on cover slips and mounted, specimen side up, on 3 by 1 inch glass slides. The mounted marrow smears were fixed in absolute methanol for 1 hour and allowed to dry.

In vitro labeling of blood and bone marrow was accomplished by incubating 1 ml. of whole blood or bone marrow aspirate with 140 μc of sodium sulfate S^{35}. After incubation for 1 hour at 37° C., the supernatant plasma containing the excess radioisotope was removed. The cells were then washed twice in cold normal saline and resuspended in unlabeled dog plasma. Smears of the final suspensions were made for autoradiographs, mounted on glass slides, and fixed in methanol.

Preparation of autoradiographs. The marrow and blood smears were dipped in NTB-3 liquid photographic emulsion (Eastman Kodak Co.), excess emulsion was wiped from the back of the slide, and the preparations were air dried for at least an hour. The preparations were stored in a low humidity atmosphere in black, plastic slide boxes at 4° C. for intervals of 15 to 32 days. The photographic emulsion was developed at 20° C. with the use of undiluted Kodak D-19 (2 minutes), 0.4 per cent acetic acid (15 seconds), undiluted Kodak fixer (5 minutes), and running tap water wash (10 minutes). The autoradiographs were stained for 30 minutes with Giemsa stain in McIlvaine-Lillie buffer[10] adjusted to pH 5.2.

Grain counting. Since S^{35} has a beta energy of 0.167 mev., the beta track range is relatively long. As a result, radioactivity in S^{35} labeled cells produced a few reduced silver grains

*Abbott Laboratories.
†Diamond Laboratories, Des Moines, Iowa.

in the emulsion outside the boundaries of the cell. However, only grains overlying a specific cell were counted. Background areas of the same size as each cell counted and at two cell diameters away from the cell under consideration were also counted. All cells of each morphologic type in an autoradiograph were examined and grains per cell recorded. Cell identification in the autoradiographs was not difficult except for separation of myeloblasts from promyelocytes. Since both of these cell types contain nucleoli and possess fine chromatin, and since cytoplasmic granules cannot be seen in the autoradiographic preparations, the classification of these cells as myeloblasts or promyelocytes was based primarily on nuclear/cytoplasmic ratio. Consequently, differentiation of these two cell types must be considered rough at best.

Calculations. For purposes of initial comparison of the degree of cell labeling, gross grains per cell versus cumulative frequency graphs were constructed (Fig. 1). In an attempt to evaluate cell labeling in a more quantitative manner, the following method was used to correct for background and establish net grain count values. A graph of the frequency distribution of grains per background area was made on arithmetic probability paper. The grain count distribution in almost all instances was roughly normal. When it was not, log probability plots were made. From these graphs the 95 per cent limits of grains per background area were determined. The value for upper 95 per cent limits for background areas was then subtracted from each cell grain count. In this manner cells which could be considered "labeled" with a high degree of confidence were selected. The proportion of cells of each morphologic type which were "labeled" was then calculated, the distribution of net grains in these labeled cells was plotted against cumulative frequency, and the median grains per labeled cell value was determined.

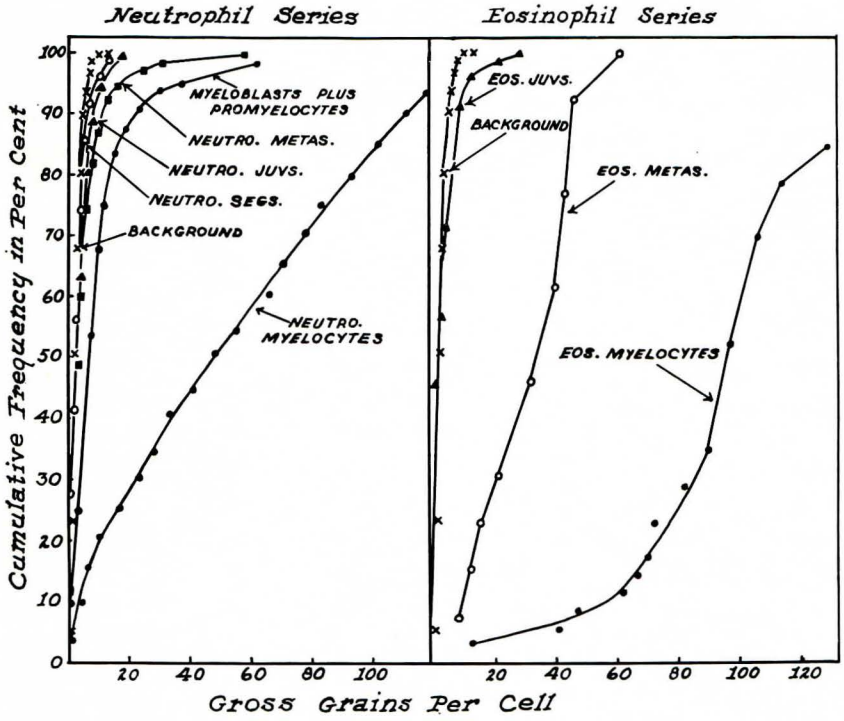

Fig. 1. Comparison of degree of labeling of various types of normal dog bone marrow cells in autoradiographs made 7 hours after the intravenous injection of 1.04 mc. per kilogram radiosulfate. *EOS,* eosinophilic; *NEUTRO,* neutrophilic; *METAS,* metamyelocytes; *JUVS,* juveniles, and *SEGS,* segmented forms.

Results

In vitro uptake of radiosulfate by dog blood and bone marrow cells. After 1 hour incubation in vitro with 140 μc per milliliter of radiosulfate, eosinophilic and neutrophilic myelocytes were heavily labeled (Table I); promyelocytes contained somewhat less label per cell and eosinophilic metamyelocytes still less (Table I). Neutrophilic metamyelocyte, juvenile, and segmented forms contained little label after this brief incubation period and the same was true of the other cell types examined with the exception of megakaryocytes.

In vivo uptake of radiosulfate by dog blood and bone marrow cells. Seven hours after the intravenous administration of radioactive sulfate (1 mc. per kilogram) heavy labeling of neutrophilic and eosinophilic myelocytes and megakaryocytes was evident in dog bone marrow (Table I). Considerable uptake of S^{35} sulfate was also noted in eosinophilic metamyelocytes (Fig. 1). The other cell types were less frequently labeled and contained considerably less label per cell. It should be noted in view of subsequent discussion that 7 hours after radio-

Table I. The relative degree of $S^{35}O_4$ uptake by dog bone marrow cells in vitro and in vivo

	In vitro labeled (1 hour incubation)			*In vivo labeled (7 hours after SO_4 injection)*		
Cell type	*No. cells counted*	*% labeled*	*Median grains/ cell*	*No. cells counted*	*% labeled*	*Median grains/ cell*
Myeloblast*	92	59	15	209	36	4
Promyelocyte*	52	77	50	68	76	14
Myeloblast + promyelocyte*	144	65	22	277	46	6
Myelocyte						
Eosinophils	20	100	58†	34	100	87
Neutrophils	219	93	65†	369	82	55
Metamyelocyte						
Eosinophils	5	60	23	13	100	35
Neutrophils	340	49	6	233	40	3
Juveniles						
Eosinophils	22	59	4	81	28	2
Neutrophils	267	23	4	301	37	2
Segmented neutrophils	26	0	0	106	26	2
Lymphocytes	43	7	3	23	13	3
Monocytes	43	21	5	4	0	0
Megakaryocytes	12	67	80	7	100	408
Plasma cells	4	50	3	3	100	20
Nucleated erythrocytes	228	4	3	218	1	1

*Because of difficulty in differentiating myeloblasts from promyelocytes in autoradiographs, it is probably better to use the combined figures.

†Some cells were too heavily labeled to count all the grains. They were excluded from this calculation and thus the median grain/cell values given are lower than the true values.

sulfate injection there was some label in promyelocytes and myeloblasts, but promyelocytes seemed to be labeled more heavily than myeloblasts (14 grains per cell); grain counts done 24 hours after radiosulfate injection revealed that median net grains per blast and per promyelocyte had decreased to 2 and 5, respectively.

Examination of autoradiographs of blood smears made 7 hours after radiosulfate injection revealed no label in any of the formed elements of the blood.

The change in marrow myelocyte radioactivity with time. The gradual decrease in marrow myelocyte radiosulfate content with time is evident in the shift in the net grain count distribution curves shown in Fig. 2. To be certain that marrow labeling by radiosulfate was reasonably uniform, two widely separated marrow sites (sternum and rib) were examined at 24, 48, 72, and 96 hours after intravenous injection of radiosulfate. The distribution of label in the several cell types was very similar in both sites. Furthermore, it can be seen (Table II) that in both sites the proportion of myelocytes labeled and the median grains per labeled myelocyte decrease gradually over many hours rather than abruptly. Even 96 hours after injection of radiosulfate, 40 per cent of the myelocytes still contained label, although the concentration per cell was reduced.

Blood granulocyte radioactivity after radiosulfate injection. The time course of blood granulocyte radioactivity in 7 dogs given radiosulfate intravenously is shown in Fig. 3. It is evident that an initial low blood granulocyte radioactivity

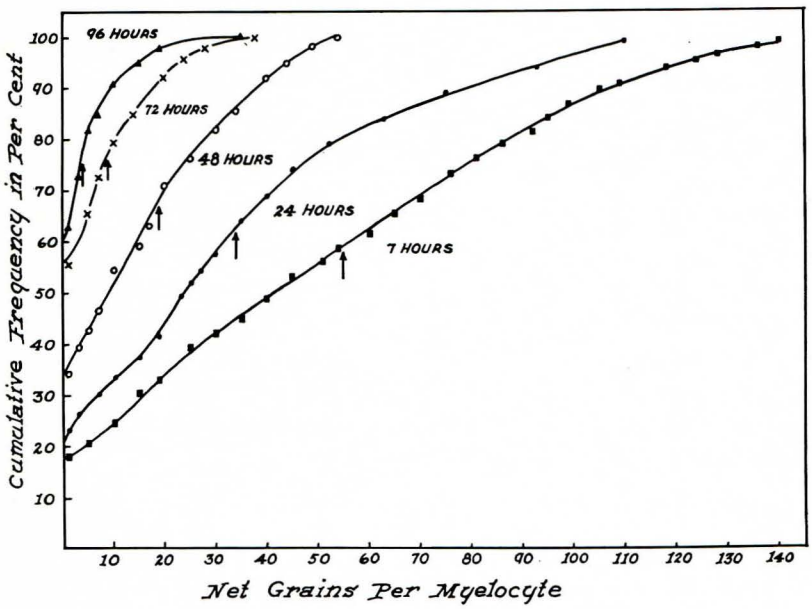

Fig. 2. The change in dog myelocyte radioactivity with time after in vivo labeling with radiosulfate. The arrows indicate the median net grains per cell value at each time period. Only myelocytes which contained more grains per cell than 95 per cent of background areas of similar size are considered "labeled" in this figure.

Table II. The change in dog bone marrow myelocyte radioactivity with time after in vivo labeling with radiosulfate

	Site A			*Site B*		
Time in hours	*No. cells counted*	*% labeled**	*Median* grains/ myelocyte*	*No. cells counted*	*% labeled**	*Median* grains/ myelocyte*
T_7	369	82	55	—	—	—
T_{24}	101	78	34	112	88	30
T_{48}	128	66	19	177	86	20
T_{72}	109	45	10	185	48	6
T_{96}	149	39	5	217	40	4

*The upper 95 percent background limits were subtracted from all myelocytes before calculating these values. Site A refers to sternal bone marrow aspiration, and Site B to rib aspiration done on the same dog at the same time.

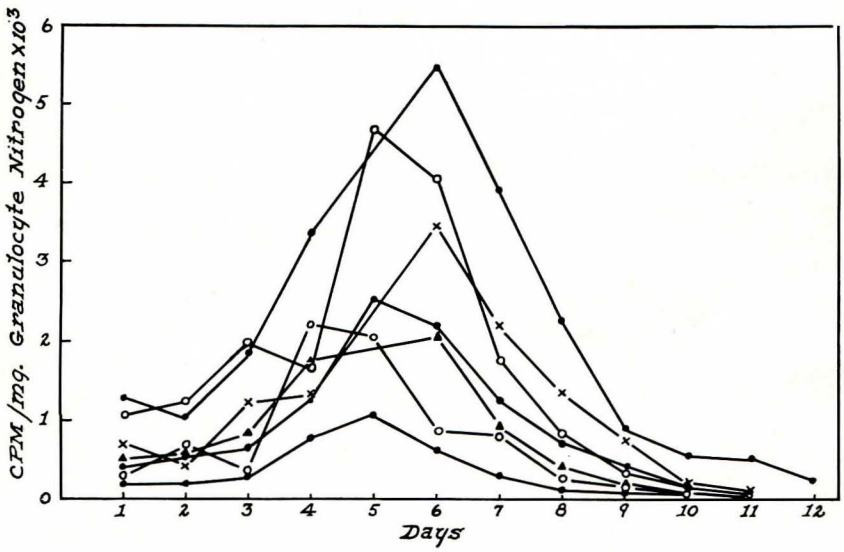

Fig. 3. The time course of blood granulocyte radioactivity values after the intravenous injection of radiosulfate.

level began to increase on the third day after label injection, reached peak values between the fourth and the sixth days and subsequently decreased, reaching background levels by the tenth to twelfth day.

Discussion

The uptake of radiosulfate by dog neutrophilic and eosinophilic myelocytes, eosinophilic metamyelocytes, and megakaryocytes both in vitro and in vivo and the relative lack of uptake in other cell forms is apparent in the present study (Table I). These findings are in agreement with similar observations on rabbits,[6] rats,[5] and man,[4, 11] wherein selective uptake of radiosulfate by myeloid cells and megakaryocytes was noted. Lajtha and co-workers described uptake of radio-

sulfate by promyelocytes as well as by myelocytes in vitro[4] and this observation is confirmed in the present study. However, under in vivo labeling conditions relatively little labeling of promyelocytes occurred as compared to myelocytes (Table I). Furthermore, by 24 hours after the sulfate injection, residual promyelocyte labeling was at a low level (median value of 5 grains per cell). These two observations make it impossible to maintain that the persistence of label in myelocytes can be attributed to feed-in of label from the promyelocyte population. Of great interest to us was the lack of label in segmented blood and marrow granulocytes initially and the subsequent configuration of the blood granulocyte radioactivity curves (Fig. 3). From these two observations it seems likely that radiosulfate initially labels primarily myelocytes in the bone marrow and these cells subsequently divide, differentiate, and are released into the blood. The blood granulocyte radioactivity curves obtained after radiosulfate injection (Fig. 3) are similar to those previously reported for radiophosphate[12] and tritiated thymidine.[13] The only difference is a somewhat more rapid return of radioactivity values to base line after radiosulfate. In dogs the bone marrow transit time, the time from label injection to peak blood radioactivity, is 4 to 6 days with radiophosphate[12] and tritiated thymidine[13] and the same appears to be the case with radiosulfate. Boggs and co-workers with the use of DFP^{32} have also estimated the mean transit time from myelocyte labeling to appearance in blood to be about five days.[14] In addition, the approximate halving of median grains per myelocyte value every 24 hours* noted in the present study (Table II) is in reasonable agreement with myelocyte generation time values of 11 to 20 hours previously reported for the dog.[15]

Since radiosulfate appears to label primarily myelocytes after intravenous injection, and little label can be detected in promyelocytes by 24 hours after injection, this label appears to offer a means for testing the hypothesis that the marrow myelocyte population is partially self-perpetuating. This hypothesis resulted from an analysis of leukokinetic data obtained after the administration of DFP^{32} to man[16] and was amplified recently by Boggs and co-workers in their report of DFP^{32} leukokinetic studies in dogs.[14] The hypothesis states that under steady state conditions the size of the normal marrow myelocyte population is maintained constant, partially by an influx of cells from the precursor promyelocyte population and partially by some myelocyte daughter cells which, after myelocyte division, remain myelocytes and can divide again while the remaining daughter cells differentiate and mature. This type of population renewal has been referred to as "semiclosed" and is to be distinguished from an "open" population in which renewal comes solely from the feed-in of cells from a precursor population.[16]

It is possible, given the initial labeling conditions and the generation time, to predict the time course of label disappearance from these two types of cell population. In a labeled "open" myelocyte population supplied by an unlabeled promyelocyte population the per cent myelocytes labeled, and the median grains

*This value is a maximum estimate of myelocyte generation time since all labeled cells were used in calculating the median grain count at each time interval. If only the most heavily labeled 20 per cent of myelocytes at each time interval are used to determine median grains per myelocyte, the grain count halving time was about 14 hours.

per labeled myelocyte would decrease from their initial values to zero in one generation time, 24 hours or less in the dog. It is apparent from Fig. 2 and Table II that this was not the case. In a labeled "semiclosed" myelocyte population with feed-in from an unlabeled promyelocyte population, the per cent cells labeled would decrease from an initial value by an amount that, in one generation time, would reflect the amount of feed-in of unlabeled cells. The median grains per labeled myelocyte would decrease to one half the initial value in one generation time. The data in Fig. 2 would appear to be more compatible with a single "semiclosed" myelocyte population than with a single "open" population of myelocytes in that label was still evident in the marrow myelocytes after 96 hours.

Are several "open" myelocyte populations, i.e., several cell divisions within the morphologically defined myelocyte population, compatible with the present radiosulfate data, and if so how many "open" populations best fit the data? A definitive solution to this question was sought by constructing a variety of mathematical models,* feeding the grain count distribution data obtained at 24 hours into each model (input data) and comparing the model output at 48 hours with the 48 hour data. This process was repeated using the 48 hour data as input and comparing the model output at 72 hours with the 72 hour data, and so on. It was hoped that by this means a single unique model of myelocyte population behavior which best fitted the data could be identified. Unfortunately, this was not possible because of the large variance around the median grains per myelocyte value and because of the difficulty in establishing values for the per cent of myelocytes labeled as time progressed. The latter difficulty arose from the fact that the grain count distribution curves for myelocytes and background areas increasingly overlapped as time progressed. The reasons for the large variance in the grain count distribution curves are not entirely clear. In any case, if we can assume that the presence of label does not alter myelocyte behavior, that reutilization and continued labeling are minimal, and that all myelocytes behave in a similar fashion, it seems evident that the myelocyte population is not of the single "open" population type. However, several "open" myelocyte populations could explain these data.

The construction of several models containing one, two, three, and four sequential "open" myelocyte divisions (or generations) indicates that four sequential "open" divisions in the myelocyte population are quite compatible with these data. The recent in vitro cinematographic studies of Boll and Kuhn[17] suggest the presence of two or three sequential divisions in myelocytes and may be interpreted as providing support for this latter model.

Could a model which assumes a nonhomogenous myelocyte population explain these data? All that can be said in this regard is that the configuration of the curves in Fig. 2 does not suggest the persistence of a population of heavily labeled cells in some sort of dormant or resting state and another population that is dividing more rapidly.

Could the persistence of radioactivity in myelocytes result from reutilization

*This part of the study was conducted with the aid of a Control Data 3200 digital computor.

of the label or continued labeling over a prolonged period? Urinary excretion and plasma radioactivity values have been reported in studies of sulfate metabolism in rats,[2] man,[7, 11] and dogs.[17] There is general agreement that from 55 to 75 per cent of radiosulfate injected is excreted in the urine in the first 24 hours. Plasma radioactivity fell exponentially to values less than 10 per cent of peak activity in 24 hours or less. In dogs the plasma radioactivity half-life was 4 to 5 hours. These data when coupled with our observation that 0.4 mc. per kilogram of radiosulfate gave undetectable myelocyte labeling and that 1.0 mc. per kilogram or greater was necessary to obtain detectable label in marrow myelocyte autoradiographs makes it unlikely that continued labeling or reutilization of radiosulfate contributed significantly to the persistent radioactivity in marrow myelocytes. In addition, since at 7 hours post injection and at all times thereafter the background grain count was less than 8 grains per area (4 to 8) and showed no trend toward significantly lower levels, the persistence of a heavily labeled marrow sulfate pool seems unlikely. It is conceivable that specific re utilization of the organic sulfate compound in myeloid cells[4] may explain the prolonged persistence of labeled myelocytes in the marrow. However, the fact that the blood granulocyte radioactivity values, as counted in a highly efficient plastic scintillation counting system rather than judged from autoradiographs, had reached background levels by 10 to 12 days (Fig. 3) would seem to indicate that radiosulfate reutilization is even less than that with tritiated thymidine.

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