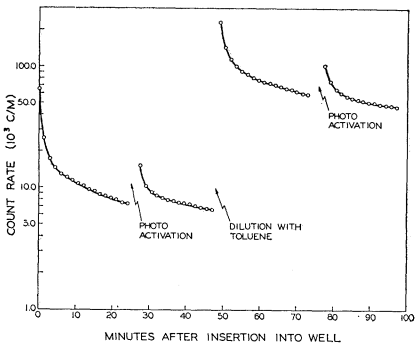

Fig. 1. Count rate of a trypsin-Hyamine solution showing the effects of exposure to light and of dilution with toluene.

Hyamine solution, prepared according to the method of Vaughan (*1*). These 100-mg protein samples usually required heating between 50° and 70° C for periods ranging from ½ hour to several hours. Samples were then cooled to room temperature, diluted with 10 ml of toluene containing 0.5 percent DPO, cooled, and counted. All counting was done with a Tri-Carb liquid scintillation counter operated at 1100 v with channel settings 6–35 and 6–100.

It was observed that neither radioactive isotope nor scintillation phosphor was needed to obtain a tremendous count rate. From 20,000 to 300,000 count/min was observed with Hyamine-protein-toluene solutions of 100-mg portions of the proteins mentioned above. This count rate decreased regularly but slowly. The normal instrumental background was reached only after several days. (For rate studies zero time was taken as start of addition of toluene to the protein-Hyamine solution.) It was also observed that protein-Hyamine solutions containing no added toluene (or radioisotope) exhibited this slow-decay phosphorescence. (For these solutions, zero time was taken as time of insertion of the cooled sample into the counting well of the counter.) In general, the magnitude of the count was not as great (up to 50,000 count/min) as it was when toluene was present. When these protein-Hyamine solutions were diluted with toluene, alcohol, acetone, dioxane, ether, water, or glycerine, the rate was raised; the first five substances yielded a count rate of several hundred thousand counts per minute. The count rate on dilution seemed to be an inverse function of the viscosity of the diluting solvent; the lower the viscosity, the higher the rate.

The count rate of both the protein-Hyamine and protein-Hyamine-solvent solutions could be increased by exposure of the solutions to an incandescent lamp. The entire phenomenon is shown in Fig. 1 for 100 mg of trypsin dissolved in 3.0 ml of Hyamine solution. Here are shown the initial count, a photoactivated count, the count after dilution with 10 ml of toluene, and another activated count. The activation consisted of exposing the sample to the light of a 75-w Reflectorspot at 25 cm for 1 minute. (Empty vials, or vials containing toluene, gave no more than a 2-count/min increase when they were so illuminated for 5 minutes.)

In all cases, the initial count increased as sample weight increased. Below a concentration of about 10 mg of protein per milliliter of Hyamine solution, the phenomenon was negligible so that the phenomenon described does not invalidate the procedure of Vaughan *et al.* for either C^{14} or H^3 counting. In several of the experiments when the protein-Hyamine solution was diluted with toluene or other solvent, the initial rate started at zero and increased within a few seconds to a maximum and decayed in the usual way. In all experiments, the difference in count rate between the two channels soon became constant and small, showing the pulse heights of the counts to be chiefly below the 35 setting. The variation of the rate with photomultiplier voltage was as follows (calling the rate at 1100 v 1.00): 1020, 0.26; 1180, 1.35; 1260, 1.28; 1340, 0.88; 1400, 0.46.

Debye and Edwards showed that proteins in alkaline solution exhibit phosphorescence (*2*). They attributed this chiefly to tyrosine and tryptophan. As a check, 100-mg portions of these two amino acids were allowed to stand with 3.0 ml of Hyamine solution until solution occurred and were then counted after addition of toluene. Both of the two gave a slight, fairly rapidly decreasing count rate: tyrosine, 1500 count/min; tryptophan, 3500 count/min. In about 10 minutes the normal background was obtained. Exposure of the solutions to the Reflectorspot gave no increase in count rate for the tyrosine; the tryptophan rate increased by 300 count/min.

Another sample of trypsin, a substance which in 100-mg portions in Hyamine solution gave a tremendous count rate, was dissolved in 1.0*M* KOH and counted. No count was obtained. Hyamine solutions without protein gave no count either alone or when diluted with toluene or when exposed to light. Hyamine solutions of sterols, or of amino acids other than those mentioned above, failed to provide counts. For a given Hyamine preparation there seemed to be a possible correlation between initial count rate and number of hydrolyzable bonds. The count rate for equal weights of glycine, glycylglycine, glutathione, Bactopeptone, and trypsin increased in the order given.

The precise cause of this phosphorescence is not known. Heating of the sample may be significant. However, the effect can be completely eliminated in several ways. As already mentioned, for small quantities of proteins the effect is inconsequential. The purity of the Hyamine itself is of prime importance. Repeated crystallization of the original quaternary chloride from toluene, until the filtrate is colorless, yields Hyamine which, when it is converted to the quaternary base in methanol solution, with small weights of nonradioactive protein and several hours of sample cooling, produces only normal instrumental background. Large weights of proteins can be used if the solution is acidified prior to counting. Acidification of a high-counting protein-Hyamine-toluene solution reduced the count rate immediately to normal instrumental background. Such acidified solutions can be counted immediately after acidification. Little or no change in counting efficiency results from acidification.

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4 April 1958

Isometric Twitch Tension of Frog Skeletal Muscle as a Function of Temperature

Current work at this laboratory on the action of high-intensity ultrasound on the twitch contraction of excised frog skeletal muscle makes it necessary to distinguish between temperature and nontemperature effects produced by the sound. Therefore a study (*1*) was undertaken to determine the variation in the magnitude of the maximum isometric tension and the action potential of excised frog biceps muscle at a number of temperatures in the range 2° to 35°C. Unfortunately, in the recent literature there appears little data on frog skeletal muscle twitch tension at temperatures above 25°C. Walker (*2*), working at the two isolated temperatures 14.5° and 27.5°C found different results for *in situ* summer and winter frogs. His experiments on excised muscles produced variable results, with an indication that twitch tension is less at the lower temperature. Hill's well-known results (*3*) are restricted to temperatures below 22°C; Buchthal's results (*4*) on single fibers are limited to temperatures up to 26°C. The work of early investigators, referenced later in this report, yielded variable results.

The following procedure was used in a series of 32 experiments. At an initial

temperature of approximately 20°C, the muscle was stimulated once every 10 seconds for a period of 2 minutes, while the action potential and isometric tension were recorded. The Ringer's solution was then siphoned from the bath container, and a solution at a new temperature was added. After an acclimatization period of 3 minutes at this new temperature, the muscle tension and action potential were again recorded. This procedure was repeated at a number of different temperatures, after which the muscle was returned to the initial temperature of 20°C to check reversibility. All muscles were tested at the experimentally determined "rest length" position. Both curarized and noncurarized preparations were studied. An RCA 5734 transducer tube, in conjunction with an amplifier and recorder, was used to measure the muscle tension. Isometric operating conditions were insured by the use of a double-spring system in association with the transducer tube shaft. A copper constantan thermocouple, inserted through the center of some of the muscles, indicated the internal temperature. The action potential was recorded by photographing the trace of an oscilloscope screen.

Electrodes, consisting of silver-plated Nichrome pins inserted completely through the muscle, were used for both stimulation and for picking up electrical responses. These electrodes, originally intended for the ultrasound experiments, were designed as follows. The end of each pin, which is fastened to a flexible lead, is covered by a plastic fitting against which the muscle rests snugly when light pressure is applied by a plastic nut which screws on the free end of a threaded pin after the pin is inserted through the muscle. This design eliminates relative motion between the muscle and each electrode, despite gross movements of the muscle. In the majority of the experiments, wherein both the mechanical and electrical responses were recorded, the stimulating electrodes consisted of a single pair of closely spaced pins rather than a series extending throughout the length of the muscle. In such experiments, the stimulus level used was one yielding maximum amplitude of action potential but not necessarily maximum isometric tension. The results obtained on the mechanical response with such electrodes were checked by a number of experiments with stimulation accomplished by a series of electrodes with an average spacing of 1½ mm along the entire length of the muscle. When this electrode arrangement was used, the stimulus level was graded, at each temperature, from a value just above threshold to one well above that yielding maximum isometric tension.

For temperatures below 20°C, the results of the experiments reported here on maximum isometric twitch tension agree with those found in the literature—that is, as the temperature decreases the amplitude of the tension increases, while the speed of contraction or shortening decreases. However, for temperatures above 20°C the amplitude of the isometric tension again increases, while the speed of contraction and relaxation also increases. The relation between the amplitude of maximum tension and the temperature is indicated in Fig. 1. The changes with temperature, over the range indicated on the graph, are reversible. Similar results were found on both curarized and noncurarized preparations. No indications of heat contractures were obtained at temperatures up to 35°C. Over approximately the same temperature range the isometric *tetanic* tension is, according to A. V. Hill (*5*), a monotonic function of the temperature, rising relatively slowly. [Hajdu's reported results on tetanic tension (*6*) exhibit a maximum within this same temperature range.]

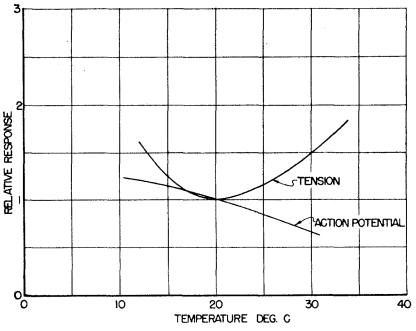

Fig. 1. Amplitude of maximum isometric twitch tension and action potential (macroelectrodes) as a function of temperature for the excised frog biceps muscle.

The electrical measurements show that the amplitude of the muscle action potential, when macroelectrodes are used, decreases monotonically with increasing temperature over the range 2.0° to 32°C. In addition, the "expected" increase in conduction velocity and decrease in duration of electrical response occur with rising temperature. These results agree with those of Sanderson (*7*), who worked with frog sartorius in the range 4° to 20°C, and with those of Welkowitz and Fry (*8*), who worked with frog biceps muscle, in the temperature range 20° to 35°C. The increase in electrical conduction velocity with increasing temperature, determined from our experiments on whole muscle, agrees very closely with the results of Wilska (*9*), who found a factor of 5.4 for the increase in velocity, for single fibers of frog skeletal muscle, as the temperature is raised from 0° to 36°C.

The fact that the action potential versus temperature relation follows a monotonically decreasing curve with rising temperature, while the amplitude of the isometric tension versus temperature goes through a minimum, is of interest, but it does *not* prove that the action potential is unnecessary for initiation of the contractile process. The amplitude of the action potential over the entire temperature range studied may always be much greater than the minimum electrical stimulus required to initiate a mechanical response.

The isometric tension curve of Fig. 1 is of interest in regard to present theories on the variation of the amplitude of twitch tension with temperature. The problem, as formulated up to the present time, has been to explain why the isometric tension decreases with increasing temperature, the highest temperatures generally considered being not much over 20°C. The explanation usually given is that at the higher temperatures the relaxation process overcomes the contraction process before full tension can be reached (*3, 10*). Since the duration of the active state is approximately halved for each 10°C rise in temperature, there is insufficient time for completion of internal shortening before decay of the active state (relaxation) begins (*11*). Theories based on such a hypothesis are inadequate for explaining the increased tension, at temperatures above 20°C, found in the authors' experiments.

Gad and Heymans (*12*), in 1890 reported results similar to those reported here on the variation of maximum twitch tension of excised frog skeletal muscle over the temperature range 0° to 30°C [see also Kaiser 1896 (*13*), Brodie 1898 (*14*), Carvallo and Weiss 1900 (*15*), de Boer 1915 (*16*)]. Unfortunately, Bernstein (*17*) attributed Gad and Heymans' results on increased tension at temperatures above 20°C to a threshold effect. The results of the experiments described here, in which multiple electrodes and supramaximal stimulation were used, definitely indicate that the increased isometric twitch tension at high temperatures is not a threshold effect. Work is now under way on a study of the form (tension as a function of time) of the isometric twitch response at temperatures above 20°C, in order to provide additional information on the behavior of isometric tension with temperature.

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1. These studies were aided by a contract (NR 119-075) between the Office of Naval Research, Department of the Navy, and the University of Illinois.
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28 February 1958

Deoxyribonucleic Acid Synthesizing Cells in Peripheral Blood of Normal Human Beings

Neoplasia, aplasia, and metaplasia are problems of great importance in medicine today. The mechanism of development of diseases involving these processes is unknown, and treatment is unsatisfactory. Perhaps this is not surprising since the mechanism of control of normal cell destruction and renewal is also obscure. The development of tritium-labeled thymidine, a specific deoxyriboside precursor of deoxyribonucleic acid (DNA), by W. L. Hughes (*1*) provided a powerful tool for the in vivo and in vitro study of cell turnover in man and animals. Individual cell histories can be followed easily using histoautoradiographic techniques (*2*). A review of earlier studies on protection against radiation-induced aplasia of the marrow (*3*) led to the hypothesis that totipotential primitive mesenchymal cells may be circulating under normal conditions. If this were in fact true, one might expect some of these cells to be actively synthesizing DNA preparatory to later mitosis. The in vitro studies reported here were designed to investigate this hypothesis (*4*).

Six healthy males 28 to 45 years of age with peripheral blood counts within normal limits were selected. Nine milliliters of blood from the antecubital vein were withdrawn under sterile conditions and placed immediately into 2 ml of a solution of normal saline containing 5 percent dextran, 2 mg of heparin, and tritiated thymidine. Dextran was used to accelerate erythrocyte sedimentation and to allow easy concentration of white cells (*5*). Tritium-labeled thymidine with a specific activity of 390 mc/mmole was added such that the final dilution was 2 μc/ml. The blood was allowed to incubate for 1 hour at room temperature, during which time the erythrocytes settled. The supernatant was withdrawn and centrifuged at 200*g* for 10 minutes. The cells were then resuspended to a concentration of 100,000/mm^3, and smears were made. The smears were fixed in absolute methyl alcohol, and stripping film was placed over the preparations as described by Pelc (*6*). The film was developed, and the autoradiographs were read after 15 days' exposure. Some smears from each sample were stained with Feulgen's stain prior to application of the photographic film; others were stained through the stripping film with Wright's stain after the film had been developed. Five thousand white cells of each individual were enumerated to determine the percentage of labeled cells. A 500-cell differential was done on each individual.

The results are summarized in Table 1. Labeled cells were found in the blood cell concentrate of all individuals studied. Between 10 and 60 grains were counted above each labeled cell. In terms of the total white cell count of the concentrate, the number of labeled cells amounted to 0.06 percent or less. A typical labeled cell is shown in Fig. 1.

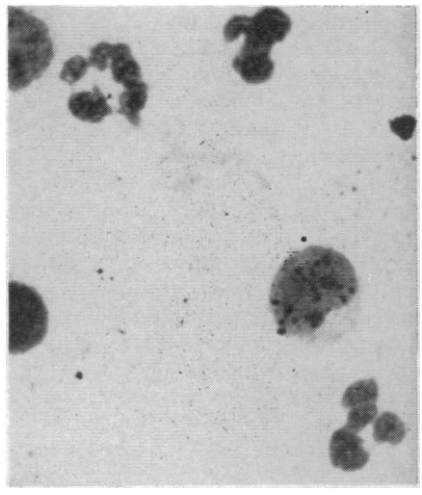

Fig. 1. Large mononuclear cell labeled with tritiated thymidine, found in the peripheral blood of a normal human being.

Table 1. Labeled cells in white cell concentrates from the blood of normal human beings.

Subject No.	Differential counts of cell concentrates (%)				Labeled cells	
	Segmented leukocytes	Lymphocytes		Monocytes	Percentage of total cells	Percentage of large mononuclear cells
		Large	Small			
1	62	7	24	7	0.06	0.4
2	78	3	16	3	0.04	0.7
3	68	11	18	3	0.02	0.1
4	52	25	21	2	0.06	0.2
5	56	13	29	2	0.06	0.4
6	67	14	16	3	0.06	0.4

Labeling was seen only in large mononuclear cells identified as monocytes, or large- and medium-sized lymphocytes. No labeled cells of the myeloid series and no labeled typical small lymphocytes were found.

The existence in the peripheral blood of man of a small percentage (but large absolute number) of circulating leukocytes that have the capacity to synthesize new DNA has been demonstrated. The synthesis of DNA is presumptive but not direct evidence that the cell is destined to divide if given proper conditions for seeding, since present beliefs are that intracellular DNA is synthesized only in preparation for cell division. We consider these cells to be predominantly the classical monocyte. Some resembled large lymphocytes. The monocyte is a phagocytic cell. We, among others, have long considered this cell to be a tissue histiocyte in transit. Does the capacity to synthesize DNA and presumably divide indicate that this cell is in reality a totipotential primitive mesenchymal cell, or does it merely indicate that this cell has the capacity to propagate itself? This fundamentally important question has not yet been answered by direct experiment. The present studies neither support nor refute the contention of Bloom (*7*), Farr (*8*), and Yoffey (*9*) that lymphocytes may be totipotential cells that are in continual transport to the marrow and become transformed into other blood cell lines. Initial in vivo studies with human beings, to whom tritium-labeled thymidine was administered, resulted in the labeling of essentially no small lymphocytes in the peripheral blood, although a small number of labeled large mononuclear cells were found (*10*).

It is clear from current studies of patients with blood dyscrasias that, with