Desoxyribonucleic Acid Synthesis by Bone Marrow Cells in Vitro

By E. Donnall Thomas and Harry L. Lochte, Jr.

EVALUATION of growth and development in bone marrow under in vitro conditions has usually been based on changes in morphology and in cell number. A basic limitation of this approach has been the limited period of survival of bone marrow cells in vitro and the difficulty of assessing the relatively minor changes in cell count and morphology that occur during this limited period of time. Isotopic methods have the advantage of permitting significant measurements of rates of incorporation of specific precursors into marrow cells over the periods of time imposed by the cultural conditions. In the course of investigations directed toward the development of suitable isotopic methods of studying bone marrow activity in vitro, a method for evaluating heme synthesis from glycine-2-C^{14} has been described.[1] Heme synthesis has the advantage of being specifically associated with erythropoiesis. However, it is a function of more mature cells during the process of maturation or differentiation rather than an evidence of growth and proliferation. In order to study proliferation it would appear worthwhile to measure a process more closely related to nuclear division or the rate of nuclear division, a process closely associated with the rate of desoxyribonucleic acid (DNA) synthesis.

In recent years a great deal of evidence has accumulated indicating that the amount of DNA per cell is quite constant.[2] It follows that the total quantity of DNA in an aliquot of tissue is an important measure of cell number and that an increase in DNA is an indication of growth. In tissue culture studies the progressive increase in the quantity of DNA as determined chemically has been used as an index of growth.[3, 4] Under some conditions this may not represent a true measurement of synthesis because of the simultaneous loss of DNA during the period of study.

Marshak[5] first showed the incorporation of P^{32} into DNA to be a good index of the rate of tumor growth. Hull and Kirk[6] found that the incorporation of inorganic P^{32} into DNA in chick embryo heart cultures parallels the mitotic coefficient and serves as a sensitive index of synthesis of new cells. Osgood[7] used the uptake of P^{32} into DNA as an index of the growth of human leukemic leukocytes in vitro and in vivo.

This present communication describes studies of DNA synthesis by bone marrow in vitro by using the rate of incorporation of C^{14}-formate into thymine as a measure of the rate of DNA synthesis. Totter[8] reported that isotopic formate is incorporated into the methyl group of thymine by bone marrow cells. Since thymine occurs only in DNA, this work suggested that the rate of DNA synthesis could be followed by direct isolation of labeled thymine without preliminary separation of ribonucleic acid (RNA) from DNA.

From the Mary Imogene Bassett Hospital (Affiliated with Columbia University), Cooperstown, New York.

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METHODS

The method of preparing aliquots of rabbit bone marrow has already been described.[1] Human bone marrow was obtained by aspiration from the iliac crest or from ribs removed at surgery. Excessive contamination of aspirated marrow by peripheral blood makes the isolation of thymine difficult. The marrow was prepared by passing it through stainless steel screens and centrifuging as described for rabbit bone marrow. Slicing of human marrow particles before passage through the screens was not found to be necessary.

The C^{14}-formate used in these studies had a specific activity of 2 μc per μM. The final incubation media contained 1 μc/ml. It also contained 250 units of penicillin and 100 μg. of streptomycin per ml. The formate, penicillin and streptomycin were pipetted into each incubation vessel, frozen, and evaporated to dryness prior to adding the bone marrow. The exact composition of the crystalloid solution was found not to be critical. In most of the studies Hanks' solution was used.

Glassware was acid cleaned without contact with detergents or dichromate. All glassware was siliconed. Twenty-five ml. Erlenmeyer flasks were used as incubation vessels.

Each aliquot of bone marrow was chosen to contain approximately 100,000,000 nucleated cells. Aliquots with less than 50,000,000 nucleated cells led to the isolation of so little thymine that accurate determination of specific activity was not possible. However, with as little as 1,000,000 nucleated cells, accurate determinations could be made by adding aliquots of carrier bone marrow to the incubation vessels at the end of the period of incubation. For most experiments, the period of incubation was 4 or 5 hours. Incubations were carried out at 37 C. with gentle shaking.

At the end of incubation the aliquots of marrow were lipide extracted and acid washed. Both DNA and RNA were then extracted from the protein residue with hot 6% perchloric acid.[2] Hydrolysis to the free bases was completed by treatment with 70% perchloric acid.[2] The details of the procedure are given below.

Lipide Extraction

At the end of the period of incubation, the bone marrow was transferred from the incubation flask into 40 ml. centrifuge tubes with three washings of cold Hanks' solution. The cells were centrifuged at 4 C. for 10 minutes at 225 x G, and the supernatant was discarded. This removed most of the serum proteins which interfere with the isolation procedure. The cells were then resuspended by agitation in 1 ml. of Hanks' solution, 15 ml. of cold 95% ethanol were added and the tubes were kept in the cold for one hour. The precipitated material was then centrifuged at 900 x G for 5 minutes and the supernatant discarded. Ten ml. of absolute ethanol were added and the tubes heated for fifteen minutes at 70 C. The tubes were centrifuged at 900 x G for 5 minutes, the supernatant discarded, and to the precipitate was added 10 ml. of absolute alcohol-ether (3:1). Tubes were then heated for 10 minutes at 70 C., centrifuged, and again treated with ethanol-ether (3:1) for ten minutes at 70 C. The precipitate was washed with 10 ml. of ether and dried for one hour in the 100 C. oven.

Acid Washing

The tubes were placed in an ice bath and 5 ml. of cold 4% $HClO_4$ with an overlayer of 1 ml. of absolute alcohol were added. The tubes were centrifuged at 4 C. for 10 minutes at 2000 x G and the supernatant discarded. The acid washing was repeated, keeping the tubes at 0–4 C. at all times.

Acid Extraction

Five ml. of 6% $HClO_4$ were added and the tubes heated at 90 C. for 15 minutes. After cooling, the pH was brought to approximately 5 with KOH. The tubes were left in an ice bath 30 minutes. After centrifuging at 2000 x G for 10 minutes, the supernatant was removed, place in a small beaker, frozen, and dried by evaporation.

Acid Hydrolysis

The dry material was transferred to a 12 ml. conical test tube, 0.2 ml. of 70% $HClO_4$ were added and the tube heated to 100 C. for one hour in a water bath. The material was then cooled, neutralized to pH 5 with KOH and allowed to stand in the cold for 30 minutes. After centrifuging at 2000 x G for 10 minutes, the supernatant was frozen and evaporated to dryness.

Chromatography

The material was dissolved in a few drops of water and put on Whatman No. 1 filter paper over a 3½ inch strip. Descending chromatography was then carried out with HCl-propanol solvent (170 ml. isopropanol, 41 ml. conc. HCl, water to 250 ml.) for 12 hours.[2] After air drying the paper, the five purine and pyrimidine bands were located under an ultraviolet lamp. The thymine band (R_F-0.76) was cut out and eluted for one hour in 0.1 normal HCl. The eluate was evaporated to dryness, put on Whatman No. 1 filter paper as a single spot and chromatographed with butanol-ammonia solvent (95 ml. of 86% aqueous n-butanol, 5 ml. conc. ammonium hydroxide) for twelve hours. The thymine spot (R_F-0.35) was again located under the ultraviolet lamp, cut out and eluted with 0.1 normal HCl.

Determination of Specific Activity

The thymine was dried on small platinum disk planchettes. The amount of thymine on the disk was equivalent to infinite thinness. The radioactivity was determined by counting in a Robinson-type flow-gas counter. The planchettes were transferred to a test tube and the thymine dissolved in 3.0 ml. of 0.1 normal HCl. The quantity of thymine was determined by observing the optical density at 265 mμ and 290 mμ in the Beckman ultraviolet spectrophotometer. Calculations were made by the following formula: Micromoles of thymine = $(D_{265} - D_{290}) \times 0.141 \times 3$. Activity was expressed as counts per minute per micromole of thymine. Additional purification by paper chromatography or ion exchange columns produced no change in the specific activity of the thymine.

It should be pointed out that in any given experiment the specific activity of the thymine will depend on the concentration of formate, on the length of incubation and on whether or

TABLE 1.—*DNA Synthesis by Normal Rabbit Bone Marrow in Various Normal Rabbit Sera*

Experimental conditions: 98 × 10^6 nucleated cells per aliquot. 50 per cent serum in Hanks'. 0.5 μM of C^{14}-formate per ml. of medium.

Serum	CPM/μM Thymine	Av. CPM/μM
1 A	16,850	
B	18,050	17,450
2 A	19,350	
B	17,800	18,515
3 A	19,850	
B	20,600	20,225
4 A	13,450	
B	11,900	12,675
5 A	11,740	
B	14,900	13,320
6 A	18,900	
B	17,150	18,025
7 A	19,800	
B	20,900	20,350
8 A	18,150	
B	18,650	18,400

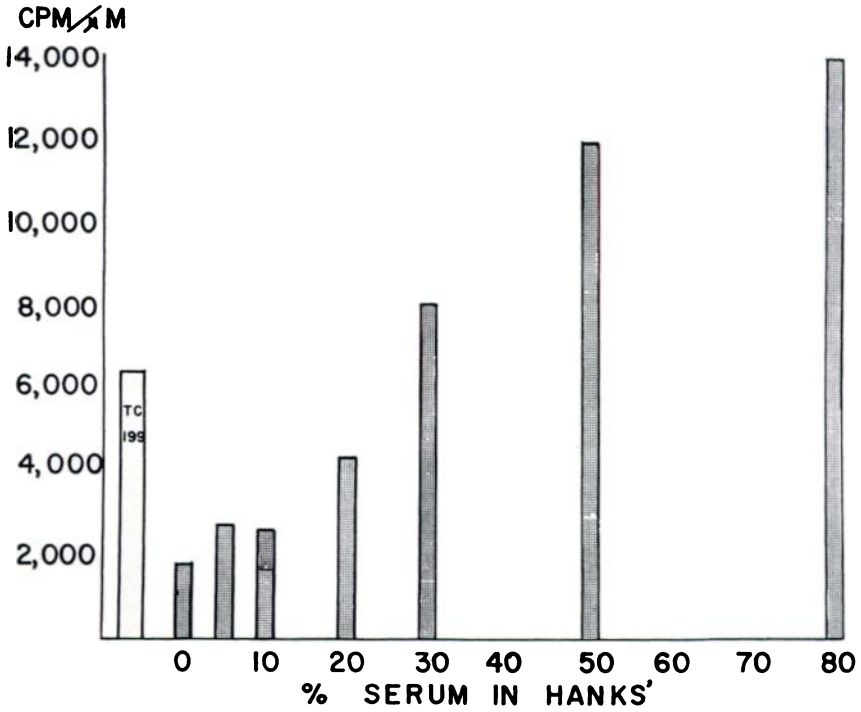

FIG. 1. The effect of various concentrations of normal serum as compared to Hanks' solution and Tissue Culture Medium No. 199. Experimental conditions: Human bone marrow, 92.7×10^6 nucleated cells per aliquot, 0.5 μM of C^{14}-formate per ml. of medium.

not carrier bone marrow was added at the end of incubation, as well as the experimental variables being studied.

RESULTS

The Reproducibility of the Method

Table 1 shows a typical experiment in which normal rabbit bone marrow was incubated in duplicate in the sera of eight other normal rabbits. That the method has been found to be quite reproducible is indicated by a standard deviation of approximately $\pm 5\%$ between duplicates. This table also illustrates the variation between different samples of normal serum with regard to their ability to promote DNA synthesis (S.D. $\pm$ 17%). Human sera have shown approximately the same variation.

The Effect of Normal Serum

Normal serum has been found to be essential for a rapid rate of DNA synthesis. Figure 1 shows the effect of various concentrations of normal serum as compared to Hanks' solution and Tissue Culture Medium No. 199.* The addition of a supplemental medium to normal serum, such as TC 199 or that described by Syverton,[9] produces a minimally significant increase in DNA synthesis. Clearly, normal serum contains important factors not present in these media.

* Difco Laboratories, Detroit, Michigan.

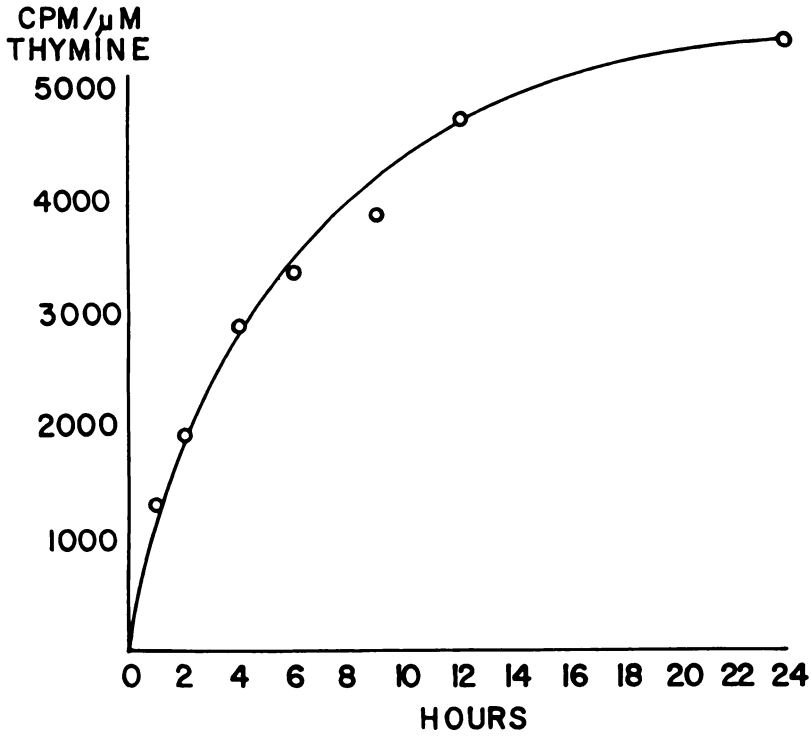

FIG. 2.—DNA synthesis as a function of time. Experimental conditions: Rabbit bone marrow in 67% normal rabbit serum in Hanks'. 105 × 10^6 nucleated cells per aliquot. 0.2 μM of C^{14}-formate per ml. of medium.

The Rate of DNA Synthesis

Figure 2 shows DNA synthesis as a function of time. The rate is almost constant during the first 4–6 hours of incubation but falls off rapidly thereafter. In most of the experiments described here, the incubation period was 4 or 5 hours. Figure 3 shows that this decrease in rate is not due to exhaustion of the labeled substrate.

The Effect of C^{14}-formate Concentration

Table 2 shows the effect of various concentrations of formate on the rate of DNA synthesis. 0.5 μM/ml. was used in most of the experiments reported here.

The Effect of pH

Figure 4 shows the effect of pH on the rate of DNA synthesis. The plotted pH values are the average of the initial pH and the pH at the end of incubation. During incubation the pH decreased about 0.1 unit. As in the case of heme synthesis, the optimal pH for DNA synthesis is on the alkaline side of the physiological pH. An initial pH of 7.6 has been used in most of the experiments listed here.

The Effect of Additional Incubation in Non-labeled Formate

Table 3 shows the effect of additional incubation in non-labeled formate. In this experiment the rabbit marrow cells were incubated in 50% normal rabbit

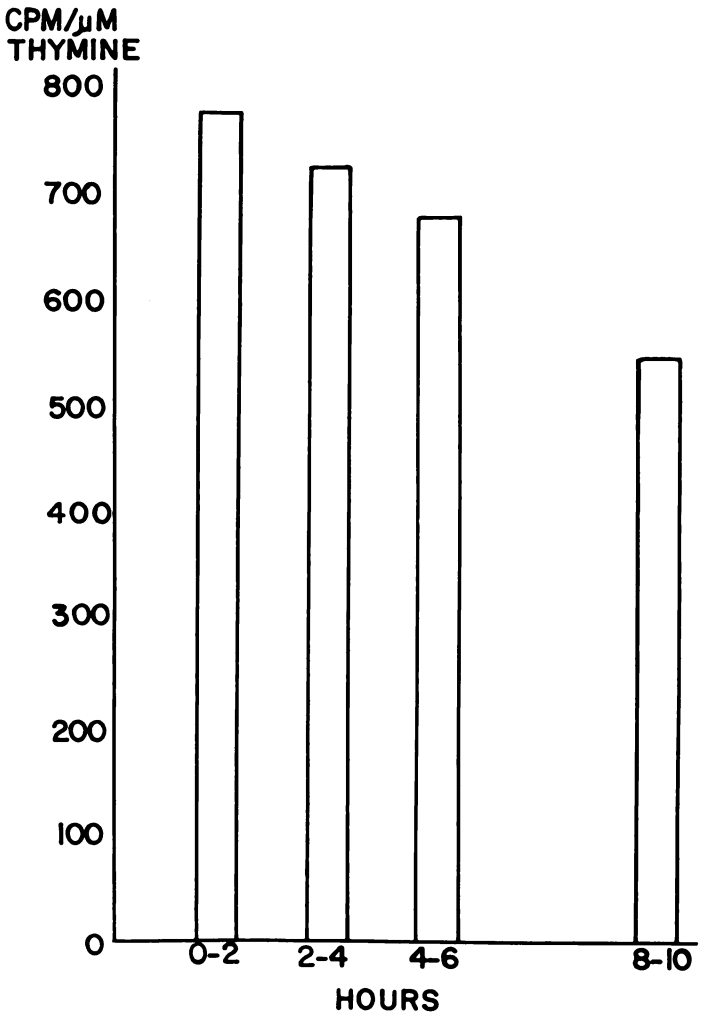

FIG. 3.—The effect of the duration of incubation on the rate of DNA synthesis. Incubations were carried out as usual, except that radioactive formate was added at 0, 2, 4 and 8 hours as indicated. Incubation with the radioactive formate was carried out for two hours. Experimental conditions: Human bone marrow in 50% human serum and Hanks'. 17.7 × 10^6 nucleated cells per aliquot. 0.5 μM of C^{14}-formate per ml. of medium. 99.5 × 10^6 nucleated cells added as carrier at the end of incubation.

TABLE 2.—*DNA Synthesis with Different Formate Concentrations*

Experimental conditions: Rabbit bone marrow in 50 per cent rabbit serum in Hanks'. 61 × 10^6 nucleated cells per aliquot.

μM Formate per ml. Medium	CPM/μM Thymine
0.1	4,720
0.3	11,375
0.5	17,450
1.0	18,200

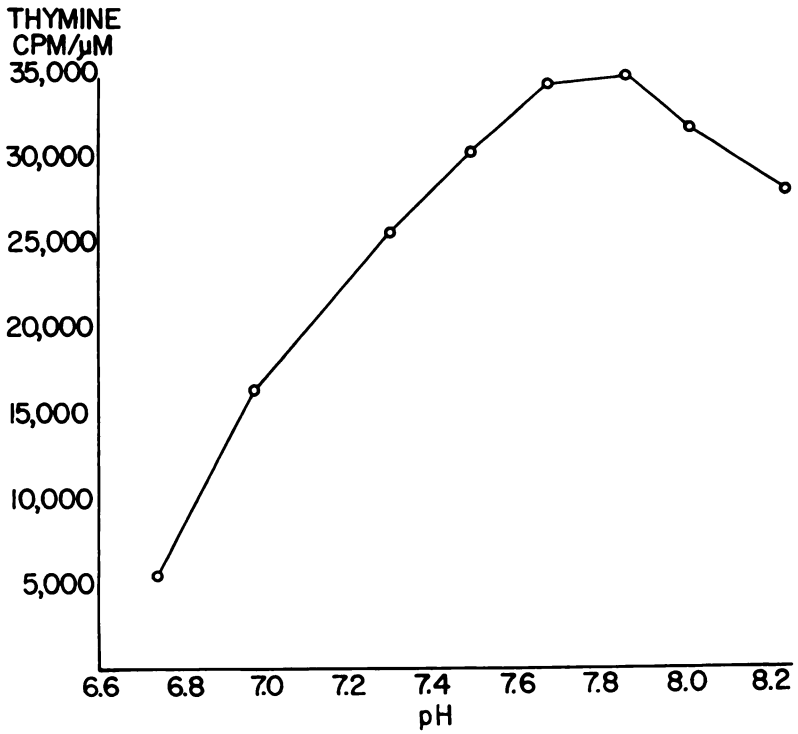

FIG. 4.—DNA synthesis as a function of pH. Experimental conditions: Rabbit bone marrow in 84% normal rabbit serum in Hanks'. 122 × 10^6 nucleated cells per aliquot. 0.33 μM of C^{14}-formate per ml. of medium.

TABLE 3. *Experimental Conditions: 95 × 10^6 Nucleated Cells per Aliquot*

Aliquot	Incubation in C^{14}-Formate (hours)	Additional Incubation in Non-labeled Formate (hours)	CPM/μM of Thymine	Average
1	0–2	none	8,640	
2	0–2	none	9,640	9,140
3	0–2	2–4	10,800	
4	0–2	2–4	11,290	11,045
5	0–4	none	13,290	
6	0–4	none	15,190	14,240
7	0–4	4–8	16,950	
8	0–4	4–8	17,750	17,350
9	0–8	none	19,050	
10	0–8	none	19,500	19,275

serum in Hanks' solution containing 0.5 μM C^{14}-formate (1 μc) per ml. for the period indicated. At the end of this period all aliquots were kept at 4 C., centrifuged 10 minutes at 225 x G, and the supernatant removed. The cells (0.1 ml.) were washed three times by resuspension and centrifugation in 5 ml. of 50% serum in Hanks' solution containing 0.5 μM of formate per ml. Then aliquots 3, 4, 7 and 8 were resuspended in the same medium and incubated for the additional periods

shown in the table. It is apparent that incubation in non-labeled substrate does not lower the specific activity of the thymine from DNA. In fact, the specific activity shows a significant increase, indicating continued synthesis from labeled compounds in the cell.

Discussion

The data presented here demonstrate that the rate of DNA synthesis by bone marrow in vitro can be followed over short periods of time and with quite satisfactory accuracy. Because of the almost constant rate of DNA synthesis over periods of approximately 5 hours, the bone marrow cells are presumed to be in a viable state approximating the normal in vivo state during this interval. During such short intervals significant changes in cell count would not be expected to occur. The measurement of cell proliferation by this method need not be synonymous with actual cell division since DNA synthesis occurs in advance of actual cell division.[2]

It has been observed that in vitro C^{14}-formate is incorporated into purines at a reduced rate while incorporation into thymine remains high.[8] One possible explanation of this phenomenon is that formate is incorporated into DNA thymine by an exchange reaction independent of DNA synthesis. If this does occur the method described here would not be a measurement of cell proliferation. It has been demonstrated that such an exchange does not occur in fibroblast cultures.[10] The data given in table 3 suggest that it does not occur under the conditions of our experiments.

The experimental data are labeled to indicate whether the experiments were performed on rabbit or human marrow. No significant difference has been found between human and rabbit marrow.

It has been of interest to compare heme synthesis and DNA synthesis. The rate of DNA synthesis decreases at a greater rate than does heme synthesis. This is consistent with the morphological observations indicating an early loss of proliferative power by bone marrow in vitro with continuation of maturation for a longer time.[11] As with heme synthesis, DNA synthesis has been found to differ from one normal serum to another. However, the rate of DNA synthesis does not show as much variation as heme synthesis in this respect.

Assessment of viability of mature cells such as leukocytes has depended on criteria such as oxidative metabolism, motility, resistance to impermeable dyes and Brownian movement.[12] In working with problems of bone marrow culture and bone marrow transplantation the criteria of viability dependent upon specialized functions of fully differentiated cells are of limited value since primary concern is with primitive cells. In this sense viability means the preservation of the cell's ability to proliferate, and therefore it would apply only to cells of sufficient immaturity to be capable of undergoing mitosis. The loss of morphologic integrity is final evidence of cell death, but since death may occur long before disruption, there is need for an independent sensitive measurement of the cell's ability to grow. Since DNA synthesis is thought to occur only in intact cells and is vitally concerned with cell duplication, measurement of the rate of its synthesis should be a valuable tool for measuring viability. In this laboratory this technic is now

being applied to the problem of assessment of viability of normal and postmortem bone marrow before and after preservation by freezing in glycerol. In such studies, it is the preservation of proliferative ability that is of greatest interest, and the rate of DNA synthesis in vitro seems to be the best available method of measuring this ability. If bone marrow retains this evidence of ability to proliferate under artificial conditions in vitro, it is presumed that it will grow at least as well and probably better when returned to normal in vivo conditions.

Summary

The incorporation of C^{14}-formate into thymine is described as a method for measuring desoxyribonucleic acid synthesis by bone marrow cells in vitro. The method is satisfactorily reproducible and permits measurement of DNA synthesis over short periods of time with small amounts of bone marrow.

Normal serum was found to be essential for rapid rates of DNA synthesis and could not be replaced by supplemented media. The rate of DNA synthesis was almost constant during the first 4–6 hours of incubation, but fell off rapidly thereafter. The ability to promote DNA synthesis was found to differ from one normal serum to another. The optimal pH for DNA synthesis was approximately 7.8.

Because DNA synthesis is vitally concerned with cell duplication, measurement of the rate of its synthesis should be a valuable tool for measuring viability of those marrow cells young enough to proliferate.

Summario in Interlingua

Le incorporation de formato a C^{14} in thymina es describite como un methodo pro le mesuration del synthese de acido disoxyribonucleic per cellulas de medulla ossee in vitro. Le methodo es reproducibile a grados satisfactori e permitte le mesuration del synthese de acido disoxyribonucleic effectuate in breve periodos de tempore per parve quantitates de medulla ossee.

Esseva trovate que sero normal es essential pro le rapide synthese de acido disoxyribonucleic. Il non poteva esser reimplaciate per medios supplementate. Le progresso del synthese de acido disoxyribonucleic esseva quasi constante durante le prime 4 a 6 horas de incubation, sed postea illo se relentava rapidemente. Le capacitate de promover le synthese de acido disoxyribonucleic se monstrava variabile ab un sero normal al altere. Le melior pH pro le synthese de acido disoxyribonucleic esseva approximativemente 7, 8.

Proque le synthese de acido disoxyribonucleic es de interesse vital in le duplication de cellulas, le mesuration de su progresso deberea esser un medio de valor in determinar le viabilitate del cullulas de medulla que es satis juvene pro proliferar.

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