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THE SEARCH FOR BIOLOGICAL EFFECTS OF ^{13}C-ENRICHMENT IN DEVELOPING MAMMALIAN SYSTEMS

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ABSTRACT

Increasing diagnostic use of stable isotopes, especially in children and pregnant women, enhances the importance of studies on the biological isotope effects in sensitive mammalian systems. Experimental data on animal systems are meager. We have studied mouse embryos at various stages and mouse limb buds in organ culture. Limb bud development *in vitro* is unaffected by incubation with 82 mol % ^{13}C-glucose as judged by either morphological or biochemical criteria. Of 271 preimplantation embryos incubated *in vitro*, 95.2% developed normally; in ^{13}C-enriched medium, 96.5% showed normal development. ^{13}C-Enrichment of the embryos *in vitro* is over 60%. Administration of 1.2 g glucose-U-^{13}C to pregnant mice during organogenesis leads to enrichment of maternal liver glycogen to over 17 mol % ^{13}C, about one-third this level in the embryo, and a lower level in maternal blood. The absolute ^{13}C content of the embryo continues to increase for several days after the end of isotope administration, while the enrichment in maternal tissues falls. The lipid fraction of the fetus is most highly labeled shortly after the end of isotope administration. These studies on developing mammalian systems have not yet revealed any alteration of normal development due to stable isotope enrichment.

There will be considerable discussion at this meeting, as at past meetings on stable isotopes, on their application to diagnostic studies on women and children, two kinds of patients which comprise a sort of metabolic *terra incognita* because of the difficulties of using radioactive isotopes in either. What is implicit in such applications is that stable isotopes are without harmful effects on the individual under investigation, even though the subject may be, in whole or in part, in a rapid stage of development. Rapidly developing mammalian systems are known to be exquisitely sensitive to changes in their environment; if there are harmful effects of stable isotope enrichment, studies on such systems should reveal them.

There are two schools of thought as to whether stable isotopic enrichment has a biological effect. The controversy resembles that on the safety of nuclear power plants. There are men of intelligence and goodwill on both sides who come to diametrically opposite conclusions. At the risk of some oversimplification, I will summarize the views at each end of the spectrum. One group holds that we already know enough, based on theoretical considerations and experimental data on simple chemical systems, to predict with complete confidence that there will be no significant biological effect of stable isotope enrichment.

Spokesmen for both groups were very much in evidence at a meeting on stable isotopes held at the University of Chicago almost exactly four years ago. The views of the other side were clear from the discussion there. For example, Dr. Alvin Tarlov of the Department of Medicine at the University of Chicago said, "I would propose that there are no data at the present time which would satisfy a board or even myself that these substances (stable isotope labeled compounds) can be given to human beings" (1). Dr. Tarlov's remarks were

seconded by Dr. Robert Levine of the Department of Medicine and Pharmacology of Yale University, who said, relative to the role of human studies committees, "I have been chairman of such a committee for the last couple of years, and I just want to say I can see no basis from any of the discussion here for any decision of any sort from such a committee. They couldn't make an informed decision" (2). Dr. Nathaniel Berlin, then of the National Cancer Institute, concluded the discussion by remarking, ".....we are going to have to face groups that will not accept theory. They want data. What are the data?" (3).

Our efforts are directed toward supplying the data. The final decision to employ stable isotopes diagnostically rests wholly in the hands of the clinicians; we are attempting to add to the factual basis for such decisions. An important fringe benefit has been to give us new tools with which to attack environmental problems of concern to our parent agency and which may also be used in stable isotope studies on the biochemistry of developing mammalian systems.

I believe the earliest attempt to study high-level ^{13}C-enrichment in a growing mammal was the mouse-feeding experiment carried out at the Los Alamos Scientific Laboratory some years ago. As most of you know, the result was that the whole-body ^{13}C-enrichment of the animals was raised to 60 mol %. These animals were apparently normal, both grossly and on microscopic examination (4). Though the overall result was striking, the statistics were inferior, and the experiment was expensive, both in time and in the amount of isotope consumed. We sought another approach which might give more statistically satisfying results without the consumption of vast amounts of enriched material. An obvious choice was the developing mouse embryo. Comparatively large numbers of animals could be used in a single experiment, and experiments

could be repeated without the consumption of large amounts of material. Both of these features, large numbers and repeatability, are absent from much of the recent work on stable isotope enrichment, both our own and that of others. These studies were done in collaboration with the Free University of Berlin; the work was divided between there and Los Alamos.

Figure 1 shows the basic approach in diagrammatic form. In one kind of experiment, mouse embryos are studied *in vitro* in the stage during which, *in vivo*, they are still free in the Fallopian tubes (that is, before implantation in the uterus has taken place). The very important period of organogenesis is studied by enriching the embryos *in vivo* during this 24-hour period in which most of the organs form and the mass of the embryo increases some 100-fold. Finally, in the most elaborate part of the study, pregnant mice are enriched with ^{13}C throughout gestation and the effects of isotopic enrichment studied, both morphologically and biochemically, in the embryos at various stages of development and in the offspring of the enriched mothers after birth. Some of the offspring in both this and the organogenesis experiment are raised to adulthood on a normal diet, then mated, and perhaps their offspring bred again, to look for an increased incidence of abnormalities attributable to alteration of the germ cells.

Some of the parameters used in evaluating the presence of abnormalities in these studies are shown in Table 1. The morphological criteria are straightforward; the biochemical assays include measurement of oxidative phosphorylation and protein synthesis and alterations in the well established patterns of ^{14}C and ^{32}P incorporation after enrichment with ^{13}C.

As these studies are still incomplete, what I will present today is a progress report. The final report may be available by the next conference

in this series. I will begin with the preimplantation embryos. This work requires that precisely timed pregnancies be produced in mice. Then, 2 days after conception, the embryos are washed out of the Fallopian tubes and incubated *in vitro* with uniformly labeled ^{13}C-glucose as their principal carbon source. The aim in such experiments is to achieve *in vitro* the same degree of development as would take place *in vivo* (that is, at the end of incubation, the embryos should have been transformed into blastocysts, at which point the embryo *in vivo* is ready to give up its wandering ways and carry out the rest of embryonic and fetal development while firmly implanted in, or at least attached to, the wall of the uterus). Figure 2 shows typical starting material for these experiments, the four cell mouse embryo. The next figure (Figure 3) shows the final product, the blastocyst.

The first question asked was simply whether the same proportion of embryos developed to the blastocyst stage in the presence of ^{13}C-glucose as did in the control. Some results are shown in Table 2. Various kinds of media were tried; some gave somewhat better results than others, but all were pretty good. The various media used are shown here, as well as whether they were enriched with ^{13}C or were natural isotopic abundance, as indicated by the superscript na. Three things can happen. Development of the embryo may terminate either at the morula stage, just before the blastocyst, indicated here by M; it may continue to the blastocyst, B; or finally, it may degenerate. The number of experiments and the number of embryos tested are also shown. The results of this rather complicated table are summarized in Table 3. Of 271 preimplantation embryos incubated 2 days in culture with natural abundance glucose, 95.2% developed normally to the blastocyst stage. Of 243 embryos incubated in the presence of 82 mol % ^{13}C, essentially the same

percentage developed normally. We conclude that incubation with the highly enriched stable isotope had no effect on development of the preimplantation embryo. The amount of carbon replaced by ^{13}C in these embryos was above 60%. This part of the experiment was fairly clearcut. The second portion of the investigation was not so satisfactory. We had hoped to return the embryos after incubation *in vitro* to the uteri of pseudopregnant foster mothers so that the remaining development would take place normally *in vivo*. Unfortunately, our results so far are not satisfactory. We have substantial losses when we try to reimplant embryos after growth *in vitro*. This is not too surprising; it is obviously a tricky business. However, it seems that embryos incubated *in vitro* with natural abundance glucose fail to implant just as often as do those incubated with the ^{13}C-enriched substrate. We have had some success, as the data in Table 4 show, but not enough. Perversely, the Ficol medium which gives the highest rate of development *in vitro* seems to give the poorest results on transplantation back into foster mothers. The ^{13}C-labeled embryos that have been successfully implanted have been examined microscopically and electron microscopically, and no abnormalities have been found in spite of the high level of enrichment; however, the numbers are too small to permit any conclusions at this point. There are ways that the transplantation efficiency may be improved, and several avenues are being actively explored.

The period of organogenesis in a mouse is between days 8 and 9 of gestation and marks the transition between embryonic and fetal life. During this interval, not only does the embryo weight increase nearly two orders of magnitude, but most of the major organ systems are formed. As one might expect, it is a period of extraordinary sensitivity to various perturbations. To enrich such embryos in ^{13}C, we starve the mothers overnight, which careful

investigation has shown to have no effect on embryonic development. Then, at the beginning of the experimental period, glucose is given intravenously or by stomach tube (at 8 g/kg), and glucose administration is repeated at 2-hour intervals for a total of 8 hours. Two hours after the last dose, some of the embryos are removed and their isotopic content determined, but the majority are left to continue development. In the long-term experiments, some of the embryos will be taken for biochemical and morphological examination just before birth; others will be born and grow to adulthood and used for subsequent mating. These experiments, like the isotope administration throughout pregnancy, will be postponed until they can be carried out in specific-pathogen-free (S.P.F.) quarters. The alternative is to risk loss of nearly half the animals from the usual animal room infections during the course of the experiment.

Our preliminary investigations have sought to determine what levels of enrichment we could expect in both the embryo and mother and how quickly the isotope administered was diluted. Some data are shown in Table 5, which indicate the partition of the isotope between maternal and fetal tissue at various periods after "pulse-labeling." The mother animal uses the initial administration of ^{13}C-glucose to rebuild her depleted supply of liver glycogen. The material responsible for the initial high labeling in liver was determined to be glycogen by nmr examination of the acid-soluble fraction and was confirmed by enzymatic and nmr analysis after hydrolysis. The maternal blood is less highly labeled initially, and throughout the experiment the whole embryo has a higher ^{13}C content than does maternal blood.

Three days after the end of ^{13}C administration, the twelfth day of gestation, the degree of labeling in maternal blood and liver has dropped sharply.

Since the mass of both tissues is unchanged, this drop is clearly due to the metabolism of ^{13}C-labeled constituents and to the transport of labeled compounds to other tissues. The drop in ^{13}C-enrichment in the embryos is much less striking, especially when one considers that, in this 72-hour period, the weight of the embryo has increased more than 600-fold. It can be calculated that the ^{13}C content of the embryo has actually increased 17-fold during this period, at the expense of label in maternal liver and blood. After an additional 4-day interval, maternal blood and liver retain appreciable enrichment. The mass of the embryo has increased about another 3-fold and is approaching isotopic equilibrium with the maternal blood. By now, the actual ^{13}C content of the embryo has started to drop; it is about one-third lower than at 12 days but is still 11-fold higher than it was initially. The implication is that the embryo is actively metabolizing some of the ^{13}C-labeled material taken up by it and that some of these metabolic products are being lost. A possible explanation is that some of the ^{13}C material is being used as an energy source by the embryo and is being converted to carbon dioxide, indicating that the embryo is not wholly dependent for its energy requirements on material passed to it across the placenta.

A somewhat similar experiment, conducted on mice in a later stage of gestation, is shown in Table 6. Here a larger amount of isotope was administered over a longer time and a chemical fractionation carried out on maternal tissues and on the whole fetus. Maternal liver is again very highly enriched in ^{13}C-glycogen, while maternal blood is about one-fifth as highly labeled -- in agreement with the data in the previous table. The acid-soluble fraction of the whole fetus, like the protein and structural carbohydrate fractions, is less highly labeled than the maternal blood, while the lipid fraction is

much more highly enriched. Thus, the lipid fraction must be largely responsible for the fact that the whole embryos are generally more highly labeled than maternal blood. We obviously have a treasure trove of labeled embryos and chemical fractions which we have only barely begun to explore.

The third portion of the experimental plan, the feeding of ^{13}C-enriched material throughout pregnancy, requires the S.P.F. quarters even more than the organogenesis experiments, since its duration is even longer. In this experiment, the starch and some of the protein in normal laboratory chow are replaced by 80 mol % ^{13}C material, to represent about 30% of the total diet. We expect that the fetuses at term will approach 20 mol % ^{13}C which, by the standards of a clinical application, is an enormous enrichment.

I will close with a description of another experimental system, originally developed by Kochhar at Virginia (5) and extended by the group in Berlin (6,7). This is the [illegible]e limb bud in organ culture. Very briefly, the limb buds are removed f[illegible] embryos at 11 days of gestation and incubated in culture. The remai[illegible] events which follow are shown in Figure 4. From a 1-mm long blob of mesenchyme (shown on the left), the bud elongates and differentiates into the structure shown on the right in which collagen deposits at the sites of the shoulder blade, the bones of the upper and lower arm, and of the hands and fingers.

Development of the limb bud is very sensitive to drug impairment, as shown in Figure 5. Here is normal development, in the center, after 7 days of culture, compared to limb buds treated with 6-mercaptopurine at two different levels. The fusing of the digits and the shortening of the long bones in the presence of the drug are unmistakable. The deformities caused by this drug *in vitro* are the same as those which result when it is administered to a pregnant animal.

Cell turnover is taking place rapidly in the limb bud in organ culture, as shown in Figure 6, giving incorporation of tritiated thymidine at various stages. Other biochemical parameters are shown in Figure 7. There is an initial drop in the amounts of protein and nucleic acid, followed by a rise. Hydroxyproline increases some 7.5-fold in culture, showing biochemically the striking increase in collagen formation which is so apparent visually. Other studies have been done on the overall metabolism of the limb bud in culture as measured by ^{14}C and ^{32}P incorporation. To make a long story short, we have cultured limb buds in the presence of ^{13}C-glucose and monitored the development of the limb buds both biochemically and morphologically. In none of the parameters investigated were any differences found between those growing in ^{13}C-enriched and those growing in unenriched glucose.

To summarize, we have examined, and are continuing to examine, a variety of mouse embryonic systems for biological effects of ^{13}C-enrichment. This effort is expected to provide a great deal of useful data for an average expenditure of about 50 g of contained ^{13}C each year for the three years of the program. In addition, the experience gained with these systems is useful in the context of other problems of interest to the U. S. Energy Research and Development Administration and, perhaps most important of all, it is likely to provide new insights into the metabolism of both the fetus and its mother.

ACKNOWLEDGMENT

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TABLE 1. Parameters of Biological Effects in Developing Mammalian Systems

I. Morphological

   A. Gross (e.g., cleft palate, skeletal deformation)

   B. Microscopic (light or electron microscope)

II. Biochemical

   A. Multienzyme systems

      1. Perturbation of ^{14}C and ^{32}P incorporation

      2. Respiration and phosphorylation

      3. Protein synthesis

TABLE 2. Development of Mouse Embryos after 48 Hours in Culture

Medium	Developmental Stage (%)			Number of Embryos	Number of Experiments
	M	B	Degree		
W-BSA-^{na}C	10	90	0	81	4
W-BSA-$^{13}C^{a}$	3	97	0	86	4
W-Fic-^{na}C	0	100	0	54	5
W-Fic-$^{13}C^{a}$	0	100	0	86	4
D-G-Fic-^{na}C	2	96	2	136	8
D-G-Fic-$^{13}C^{a}$	0	92	8	71	5

[a] 100 mg/100 ml glucose-U-^{13}C, 82 atom % ^{13}C.

TABLE 3. Development of Mouse Embryos after 48 Hours in Culture

Number of Embryos	Medium	Blastocysts (%)
271	Natural abundance	95.2
243	82 mol % ^{13}C	96.3

TABLE 4. *In Vivo* Development of Cultured Embryos

Medium	Transplanted Embryos (day 18)	
	Resorbed	Living
W-BSA-^{na}C	2	6
W-Fic-^{na}C	12	2
W-BSA-^{13}C	2	6

TABLE 5. ^{13}C-Enrichment of Fetal and Maternal Tissues after 10 Hours Isotope Administration to Pregnant Mice[a]

Days of Gestation	Source	Atom % ^{13}C
9	Maternal liver	17.40
	Maternal blood	3.59
	Whole embryo (0.1 mg)[b]	4.98
12	Maternal liver	3.32
	Maternal blood	1.46
	Whole embryo (66 mg)[b]	2.10
16	Maternal liver	1.85
	Maternal blood	1.33
	Whole embryo (173 mg)[b]	1.35

[a]Glucose-U-^{13}C (83.8 mol % ^{13}C) was given by stomach tube between days 8.5 and 9 of gestation. A 30-g mouse received 1.2 g of glucose, or about 480 mg ^{13}C.

[b]Dry weight.

TABLE 6. ^{13}C-Enrichment of Fetal and Maternal Fractions after 48 Hours Isotope Administration to Pregnant Mice[a]

Source	Fraction	Atom % ^{13}C
Maternal liver	Acid-soluble	34.20
Maternal blood	Acid-soluble	7.90
Whole fetus	Acid-soluble	5.89
Whole fetus	Lipid	17.50
Whole fetus	Glucosaminoglycan	4.38
Whole fetus	Protein	4.00

[a]Glucose-U-^{13}C (80 atom % ^{13}C) was given by stomach tube between days 10 and 12 of gestation. A 30-g mouse received 1.6 g of glucose, representing about 500 mg of ^{13}C.

Figure 1. A diagrammatic representation of studies in progress on the effects of ^{13}C-enrichment on various stages of mouse development. Interval #1 includes studies *in vitro* and *in vivo* on the preimplantation embryo. Interval #2 represents enrichment of the fetus during organogenesis, while the third interval represents isotopic enrichment throughout the prenatal and perinatal periods. The search for abnormalities induced by ^{13}C-enrichment includes morphological and biochemical investigations at various levels.

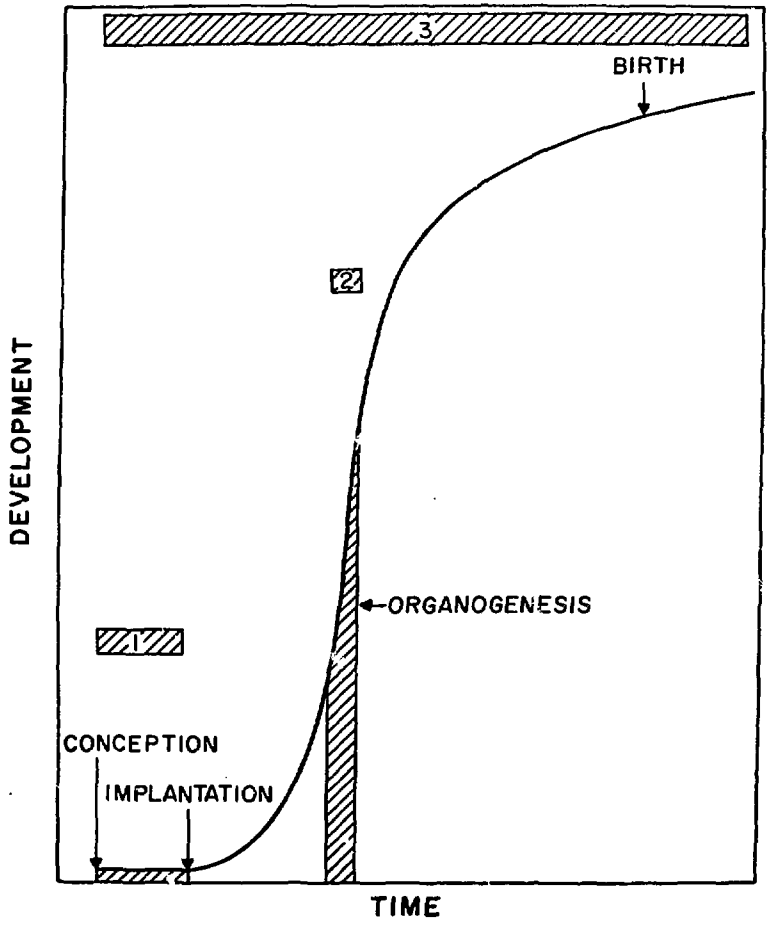

Figure 2. The preimplantation mouse embryo at an early (four-cell) stage. This is the starting material for the *in vitro* incubation with ^{13}C-labeled glucose.

Figure 3. The mouse blastocyst in the terminal stage of preimplantation development. Subsequent to this, the embryo implants in the uterus where subsequent development takes place.

Figure 4. Development of the mouse limb bud in organ culture. The limb bud is removed from 11-day-old embryos and placed in culture. At the end of 6 days in culture, the limb bud has greatly elongated and differentiated. The collagen deposition at the sites of the scapula, humerus, radius, and ulna and in the bones of the hand and fingers is clearly seen [from Neubert *et al.*, 1974 (6)].

Figure 5. Mouse limb buds after 7 days in culture. Controls are shown in the center and the effects of two levels of 6-mercaptopurine (6-MP) at right and left. Syndactyly and phocomelia (fusion of the digits and shortening of the long bones) can be seen in all limb buds treated with 6-MP for 5 days in culture [from Neubert *et al.*, 1974 (7)].

Fig 5

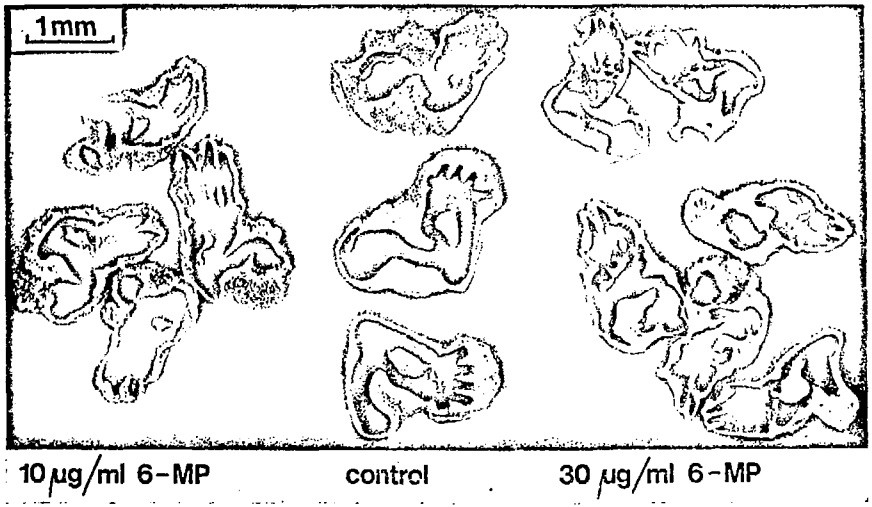

Fig. 5. Mouse forelimb buds after 7 days in culture in the presence of 6-mercaptopurine (6-MP). The limb buds were transferred into culture on day 11 + 2 hrs of gestation. After 24 hrs allowed to the explants to adapt to culture conditions the medium was replaced by another one containing 6-MP. After the third day this medium was exchanged for a fresh one containing the same inhibitor concentration. "Syndactyly"-and "phocomelia"-type malformations can be seen at all the explants with both concentrations of 6-MP

Figure 6. Thymidine incorporation into DNA of limb buds in organ culture. Tritiated thymidine was added at the beginning of a 24-hour period (arrows). Rates of incorporation for the various intervals are shown at the top [redrawn from Neubert *et al.*, 1974 (6)].

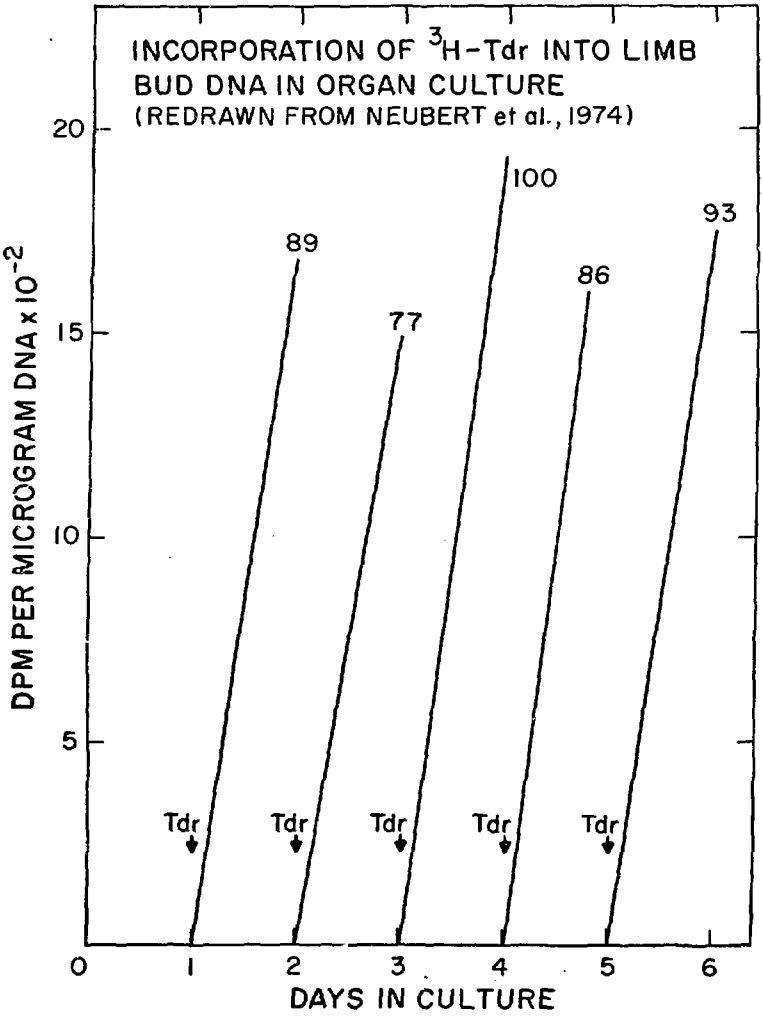

Figure 7. Some biochemical parameters of mouse limb bud development in organ culture. The DNA content increases only 10% in culture, RNA increases about 45%, and protein doubles. The content of hydroxyproline increases some 750% [redrawn from Neubert *et al.*, 1974 (6)].