EUR 3290 f-e

STABILITÉ CHIMIQUE DES BASES PYRIMIDIQUES TRITIÉES ET QUELQUES PROBLÈMES POSÉS PAR LEUR UTILISATION EN BIOLOGIE

CHEMICAL STABILITY OF TRITIATED PYRIMIDINE NUCLEOSIDES AND SOME PROBLEMS RAISED BY THEIR USE IN BIOLOGY

EURATOM

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COMMUNAUTÉ EUROPÉENNE DE L'ÉNERGIE ATOMIQUE
EUROPEAN ATOMIC ENERGY COMMUNITY

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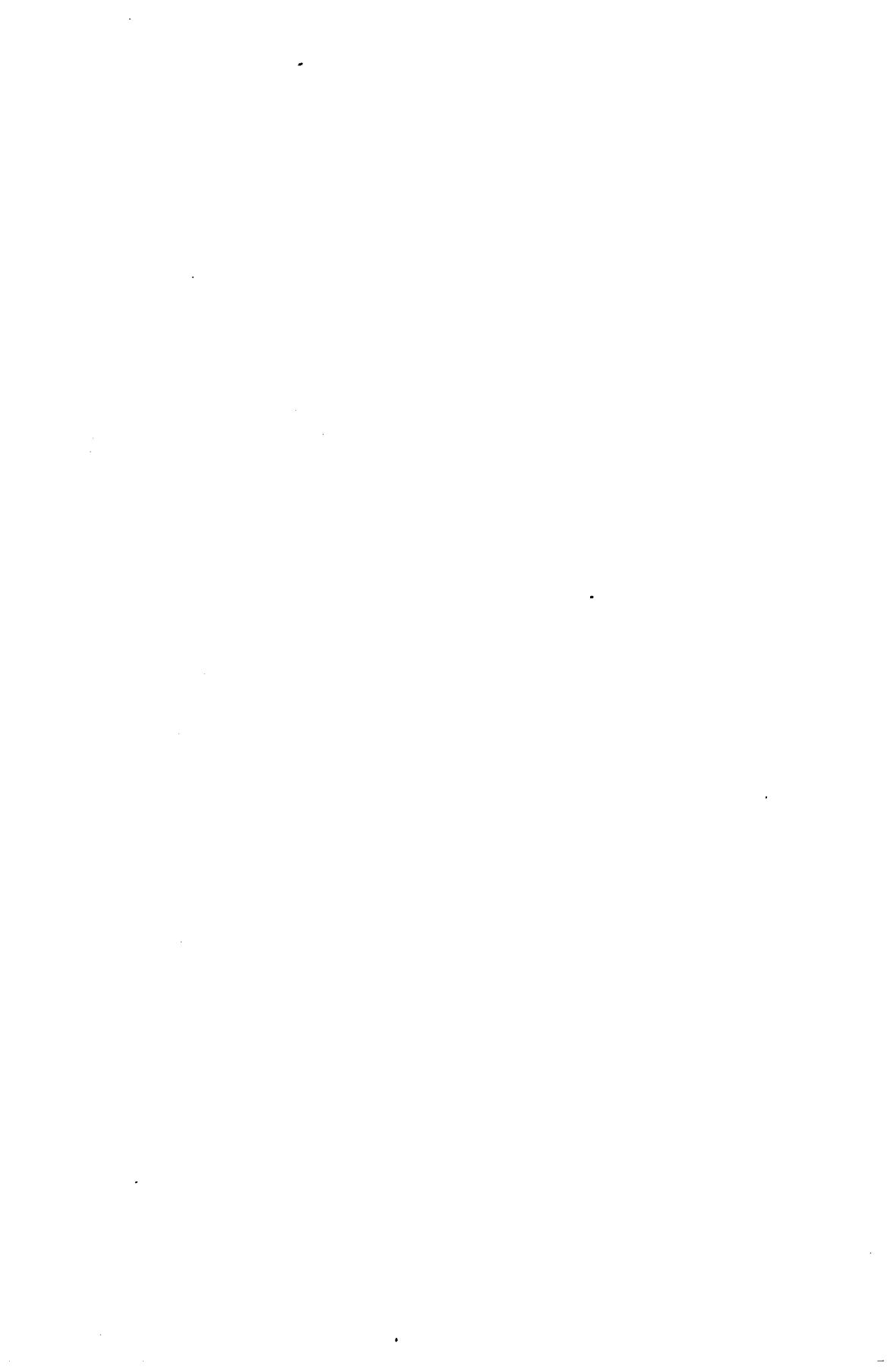
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Actes du Colloque tenu à MOL/Belgique, 16-17 mars 1965
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EDITORS'NOTE

The utilization of tritiated thymidine in biological experiments as a label for DNA has produced a number of unexpected results such as cytoplasmic labelling of abnormally low nuclear labelling. Some of the results could be ascribed to purely biological causes, among which dilution on the marker by the endogenous pool or incorporation of labelled degradation products are the main ones. It was felt, however, that the position of the tritium on the thymidine molecule also played an important role and, because of the purely chemical character of this question, it appeared useful to bring chemists and biologists together to discuss what their respective problems were and how the requirements of the latter group could be met by the former. This was the aim of the Mol Colloquium Symposium, whose proceedings are reported in this volume, and whose programme dealt with methods of preparing tritiated thymidine, storage conditions, and problems involved in its biological use. These proceedings contain evidence that both the nature and the position of the label on the thymidine molecule are indeed important.*

The Editors.

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* A publication by Bryant which appeared after this Colloquium Symposium (*J. Cell Biol.*, 1966, **29**, 29) reports that in mouse tissues, 1 to 10 % of the tritium from injected thymidine labelled on the methyl group is bound to the proteins, owing to transmethylation reactions.

WELCOME ADDRESS

J. GOENS

General Manager of CEN, Mol, Belgium

Ladies and Gentlemen,

It is with great pleasure that I welcome here at CEN the participants at the colloquia on tritiated purine and pyrimidine nucleosides and on the catabolism of pyrimidines. These were organized, as you know, under the joined sponsorship of Euratom and CEN, and I should wish to thank our partners for having entrusted us with its local organization. My thanks also go to the organizers, Messrs. Sirchis from Euratom, Goutier and Winand from CEN. The present colloquia have for us a special meaning that indicate that after a modest beginning less than 5 years ago, our Radiobiology Department is coming of age. Its place among our other research Departments is now well established as well as the fact that all the developments of nuclear energy for peaceful purposes imply an increase in the basic knowledge of the interactions between irradiation and living organisms. It is indeed there that the basic facts can be found from which practical data such as the tolerance level may then be derived. Radiobiology, in this way, makes an important contribution to the industrial applications of nuclear energy. But nuclear energy has since long repaid in advance its debt to Biology by putting at its disposal these very important research tools which are the radioisotopes and the labelled molecules. Your work, during the 2 days to come falls, I understand, more into this last category and, as an engineer who fully realizes the importance of it, I wish you an interesting and fruitful meeting.

MÉTHODES DE PRÉPARATION
DE LA THYMIDINE TRITIÉE

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INTRODUCTION

Depuis près de 15 ans déjà [1], la thymidine marquée est utilisée comme traceur dans les travaux biologiques. Marquée avec le tritium, elle est encore l'un des traceurs les plus utilisés actuellement.

Avant d'aborder les études biologiques, il semble très utile de rappeler les différentes méthodes employées pour la tritiation de ce nucléoside. La connaissance des méthodes de préparation, des tests de pureté effectués, de la stabilité du marquage, permettra à l'utilisateur, biologiste ou biochimiste, de connaître les qualités et les défauts de son traceur. Une interprétation erronnée des résultats pourra ainsi, dans certains cas, être évitée.

EXAMEN DES DIFFÉRENTES POSSIBILITÉS DE MARQUAGE
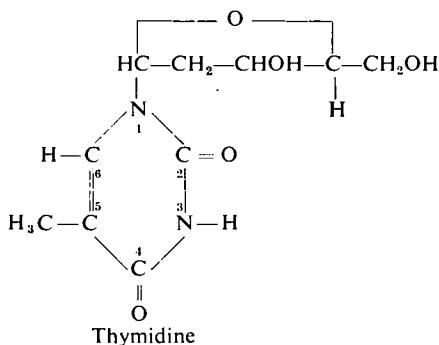
Thymidine

FIG. 1

Tous les atomes d'hydrogène de la thymidine occupent des positions stables, à l'exception de deux hydrogènes hydroxyliques du désoxyribose et de celui du groupe imidé de la portion pyrimidique.

Un marquage total ou partiel du désoxyribose présente toutefois un désavantage. En effet, des réactions de transfert du désoxyribose donneraient naissance à d'autres désoxyribonucléosides tritiés. De plus, l'oxydation du désoxyribose tritié produirait du ribose tritié qui participerait à la synthèse de l'ARN, ce qui ferait perdre l'énorme avantage du caractère hautement spécifique de la thymidine.

Les seules positions intéressantes de marquage sont donc : sur le carbone en 6 et sur le groupe méthyle en 5.

MÉTHODES DE MARQUAGE

D'une façon générale, on peut classer les différentes méthodes de marquage en deux groupes distincts :

1° Méthodes basées sur l'échange;

2° Méthodes basées sur la synthèse.

MARQUAGE PAR ÉCHANGE

En présence d'un catalyseur, une réaction d'échange isotopique entre la thymidine et le tritium, l'eau tritiée ou l'acide acétique tritié donne la thymidine tritiée [2-4]. Les rendements d'échange, sur des positions stables, sont cependant très peu élevés. Pour obtenir une radioactivité spécifique de 500 mCi/mM — radioactivité encore relativement moyenne — Murray et Petersen [4] ont utilisé 5 ml d'eau tritiée dont la radioactivité était de 2 700 curies ! Dans de telles conditions, une hydrolyse très importante se manifeste et, plus grave encore, une grande partie de la radioactivité se retrouve dans le désoxyribose.

Murray et Petersen [4] ne nous renseignent pas sur la proportion de radioactivité dans le désoxyribose, mais les enregistrements des chromatogrammes effectués après 14 mois de conservation nous donnent une idée à ce sujet [5]. En effet, les radioactivités relatives de la thymine et du désoxyribose d'hydrolyse indiquent une radioactivité de plus de 50 % dans le groupe désoxypentose de la thymidine.

MARQUAGE PAR SYNTHÈSE

La synthèse chimique de la thymidine semblant tout à fait impossible, le greffage du désoxyribose sur la thymine se fait par réaction enzymatique.

La thymine tritiée pour cette réaction enzymatique peut être obtenue par échange ou par synthèse. Par échange, la thymine tritiée présente une radioactivité d'environ 75 % en 6 et 25 % sur le méthyle.

Par synthèse, la thymine s'obtient spécifiquement marquée sur le méthyle par réduction d'un halogénométhyle ou hydroxyméthyluracile.

De nombreuses variantes de la réaction enzymatique T → Tdr ont, à ce jour, été décrites dans la littérature [6-11].

La figure 2 montre la composition d'un milieu d'incubation utilisé pour cette synthèse. Le donneur de désoxyribose est la désoxyuridine, tandis que l'enzyme catalyseur est préparé extemporanément à partir de foie de rat. La réaction se fait à une température de 37 °C pendant une heure.

Ce milieu étant exempt de thymidine inactive, la radioactivité spécifique de la thymidine tritiée est exactement celle de la thymine tritiée de départ. Nous avons choisi la chromatographie sur papier comme technique de purification.

La figure 3 montre la photographie obtenue en lumière U. V. d'un chromatogramme préparatif (environ 0,1 mM) ainsi que son enregistrement radioactif. Ce premier système (butanol-n saturé par H_2O : NH_4OH 25 % 4/1) permet d'isoler

Composition du milieu d'incubation

Substance	Quantité		Volume ml
	mM	mg	
Sucrose	22	7 500	
Tampon phosphate pH 8	0,090		125
Tampon Tris (Tham)	0,950	119	
Chlorure de potassium	0,430	32	
Chlorure de magnésium	0,125	12	
Malate de potassium	0,500	105	75
Glutamate de potassium	0,500	112	
Pyruvate de potassium	0,500	63	
Déoxyuridine	0,400	91	
Adénosine triphosphate	0,125	63	10
Thymine ³H	0,200	25,2	10

FIG. 2.
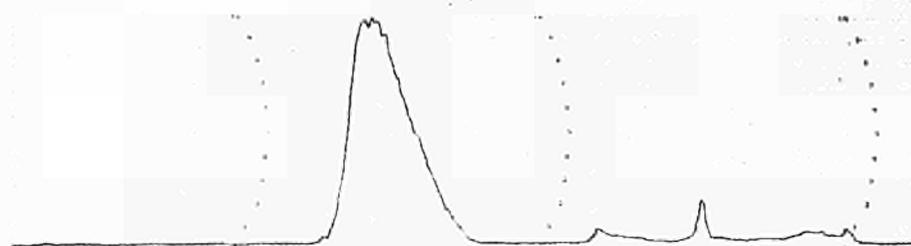
Thymine-thymidine   Désoxyuridine
Uracile   Sucrose
FIG. 3.   ATP

quantitativement le mélange thymine-thymidine, de tous les autres composés du milieu enzymatique.

Un deuxième système (acétate d'éthyle, acide formique, eau 60 : 5 : 35) permet l'extraction de la thymidine tritiée et la récupération de la thymine tritiée. C'est ce que nous montre la figure 4.


Thymine   Thymidine   Origine
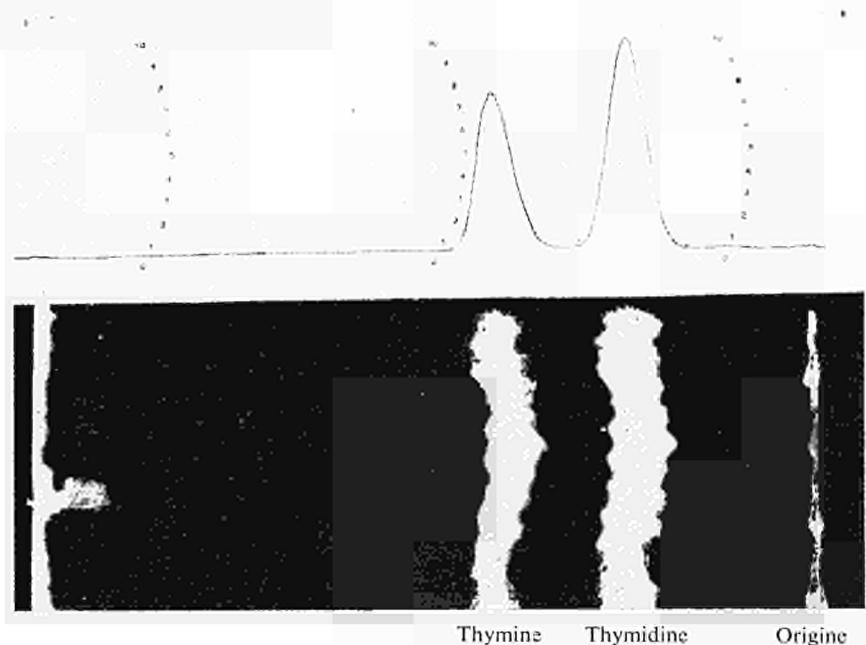
Fig. 4

Ces méthodes de purification donnent toute garantie de pureté radioactive qui est supérieure à 99 % comme le prouve la figure 5.

La pureté chimique est contrôlée par spectrométrie U. V. et les spectres obtenus sont, en général, meilleurs que ceux des produits inactifs du commerce.

Une observation très importante peut être faite à la suite de cette synthèse. La radioactivité spécifique de la thymidine tritiée obtenue est exactement la même que celle de la thymine de départ. En plus de la stabilité chimique du marquage, la thymidine tritiée passant avec succès les épreuves acide et alcaline, on doit lui reconnaître une parfaite stabilité dans un milieu biologique tel que celui d'incubation utilisé.

MÉTHODE DIRECTE DE PRÉPARATION

Depuis quelque temps, nous utilisons une méthode directe de préparation de la thymidine-méthyl-^{3}H. La réduction de la 5-hydroxyméthyl-désoxyuridine par l'hydrogène tritié donne, en effet, directement la thymidine-méthyl-^{3}H.

La synthèse est extrêmement rapide et la purification se fait en une seule chromatographie sur papier.
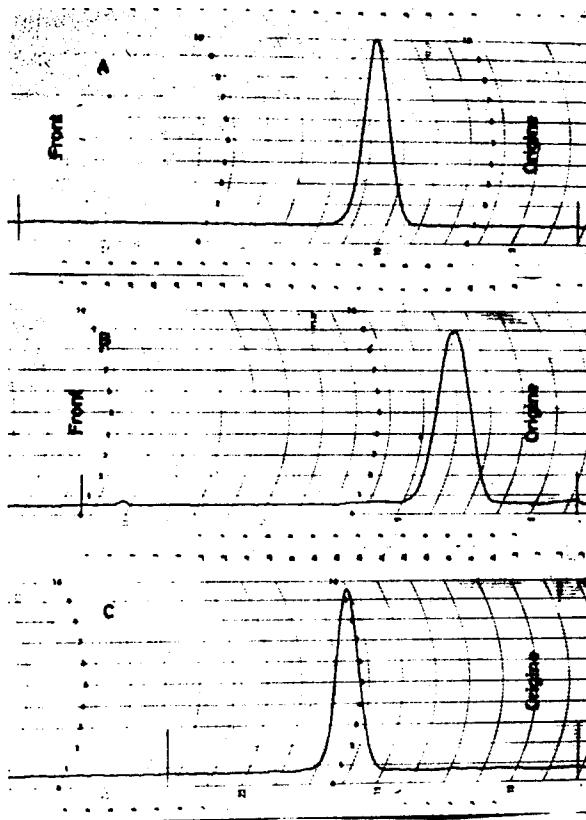
FIG. 5. Enregistrement des chromatogrammes de thymidine tritiée.

Solvants : A. Butanol N saturé avec NH_4OH 25 % — H_2O 1 : 4 V/V
Rf : 0,38
Rf Référence (sigma) : 0,38
B. Acétate d'éthyle-acide formique-H_2O 60 : 5 : 35
Rf : 0,25
Rf Référence (sigma) : 0,26
C. Acétate de butyle-acide acétique-butanol N-H_2O 3 : 1 : 2 : 1
Rf : 0,58
Rf Référence (sigma) : 0,56.

DISCUSSION

De l'étude de ces différentes méthodes de préparation de la thymidine tritiée, il apparaît que l'on peut marquer ce désoxynucléoside spécifiquement sur le méthyle en 5. En ce qui concerne la thymidine 6³H, le problème est tout différent. En effet, les thymidines dites 6³H du commerce sont, en fait, obtenues par échange ou préparées à partir de thymines tritiées, elles-mêmes obtenues par échange.

Il existe bien sûr des méthodes qui permettraient d'obtenir un marquage spéci-
fique de la thymine en 6. Il semble toutefois que ce marquage nécessite obligatoire-
ment la construction du cycle pyrimidique. Il faut bien avouer que l'on a, depuis
longtemps, perdu l'habitude de synthèses aussi complexes dans les marquages avec
le tritium. En effet, la possibilité de marquages directs est l'un des avantages les
plus appréciables du tritium. Je vous soumets comme exemple, une méthode de
marquage exclusif en 6 que nous avons utilisée avec succès.
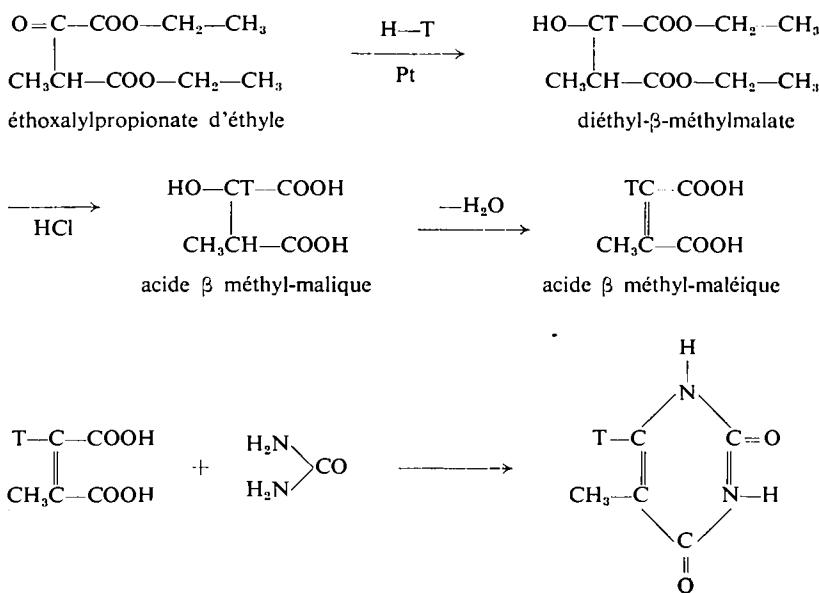
Cette méthode présente l'inconvénient de donner d'assez mauvais rendements,
aussi bien dans la réduction de la fonction cétonique que dans la condensation
avec l'urée.

CONCLUSION

Nous disposons actuellement de méthodes très simples pour la préparation
de la thymidine généralement tritiée, marquée uniquement dans sa portion pyrimi-
dique et spécifiquement sur le méthyle. Les autres possibilités sont la thymidine
marquée uniquement sur le désoxyribose et spécifiquement en 6.

Chacune de ces formes de marquage présente peut-être un avantage pour cer-
tains travaux, mais ce qui est plus important est que l'une ou l'autre de ces formes
pourrait présenter de graves inconvénients.

Des effets isotopiques peuvent apparaître et devenir très importants pour des
positions particulières de l'isotope. Une labilisation du tritium peut se produire dans
certaines conditions, telle la perte de l'hydrogène en 6 de la thymidine sous l'action
de la thymine-oxydase signalée par Pastore et Friedkin [12].

Nous attendons des biologistes des renseignements tels que ceux-ci, de façon à connaître les caractéristiques exactes que doivent présenter les traceurs que nous préparons à leur intention.

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DISCUSSION

EVANS : I would like to ask Dr. Winand if he intends to degrade the methyl labelled thymidine and to see if there is any tritium in the 6th position by this method.

WINAND : No, but I think that with the reduction of the cetone group it is not possible to have a label on the hydroxyl.

EVANS : No, I mean the reduction of your hydroxymethyldeoxyuridine.

WINAND : I think that there is no difference between the reduction of hydroxy-methyldeoxyuridine and hydroxymethyluracil.

EVANS : Even under the catalytic conditions you don't think that there is any tritium in the 6th position. Yet, I find this would be a little bit surprising that there is not some in the 6th position (even if just a few %) because we did an experiment in Amersham stirring thymidine in solution with tritium gas, and found that there was incorporation of tritium in the 6th position just a little, only 1 or 2 %. So you may under the catalytic conditions still get a few %.

WINAND : I did not try degradation of this product, but I made a comparison between the reduction of hydroxymethyluracil and this product. I think that in the case of tritiated thymine made by the reduction of hydroxymethyluracil there is no labelling in the 6th position. I don't see differences between the two reductions.

EVANS : You have proved that there is no difference between the two reductions ?

WINAND : No.

EVANS : You assume that there is no tritium now in the 6th position but it would need to substitute the 6th position with something or to degrade the molecule to prove it.

WINAND : I think it is very easy to prove, for example by oxydation with thymine oxydase and see if by a measurement of the specific activity a part was in the 6th position.

ALTMAN : I would like to ask what is your yield in the synthesis ?

WINAND : I think that in such reduction as reduction of hydroxymethyldeoxy-uridine and hydroxymethyluracil it is of no importance to obtain a good yield, because the tritiated compound is different from the precursor. The problem of specific activity is the biggest problem, and not the problem of yield.

PICHAT : Quelles sont les activités spécifiques que vous obtenez par réduction de la 5-hydroxyméthyl-désoxyuridine ?

WINAND : Je dois dire d'abord que je n'ai pas essayé d'avoir une activité spécifique maximum. Je prends cette précaution-là. C'est-à-dire que nous avons obtenu une activité maximum de 10 curies par millimole, et en fait cette activité-là nous a surpris, mais elle était déjà plus grande que ce que nous prévoyions et ce dont nous avions besoin ; je crois qu'il est possible d'aller plus loin encore, mais cela ne présente aucun avantage.

PICHAT : Nous employons précisément la même méthode, mais nous avons été moins habiles que vous parce que le maximum que nous ayons obtenu c'est 5,8 curies par millimole. Mais habituellement l'activité est aux alentours de 2,5 à 3 curies par millimole.

WINAND : Je sais qu'il y a de grandes difficultés parce que le grand problème de cette réduction est de trouver un bon solvant pour ce produit. Il est normal, d'après la constitution de ce produit, qu'il soit très soluble dans l'eau, dans l'alcool, mais ce sont précisément des solvants qui ne conviennent pas et je crois que vous utilisez la même chose, c'est-à-dire comme solvant : 50 % d'eau, 50 % d'acide acétique. Evidemment il y a une perte énorme. Mais je crois que si vous mettiez un peu plus de tritium vous arriveriez à des activités spécifiques plus élevées. C'est une question de rapport, disons de quantité de tritium et de quantité de solvant, le produit étant négligeable.

PICHAT : Et je crois néanmoins que, dans tout procédé catalytique d'introduction du tritium dans la thymidine, on ne peut pas assurer, sans avoir fait la dégradation, qu'il n'y a absolument pas de tritium en position 6 ou inversement, si vous aviez un chrome en position 6 et que vous le substituiez par du tritium, vous ne pourriez pas assurer qu'il n'y a pas du tout de tritium dans le groupement méthyle, à moins d'en faire la preuve expérimentale.

WINAND : Non, j'ai simplement fait une relation entre les deux réductions et comme je crois que la preuve a été faite, ou à peu près, que dans le cas de la réduction d'hydroxyméthyluracile il n'y avait pas de marquage, du moins supérieur à 2 %, en fait je ne vois pas du tout la différence entre les deux produits, car la seule différence est une différence de poids moléculaire.

PICHAT : Maintenant je suis tout à fait d'accord avec M. Winand lorsqu'il dit que c'est aux biologistes de nous dire ce qu'ils veulent, s'ils veulent de la thymidine marquée sur le méthyle ou de la thymidine marquée en position 6. Nous espérons que ce Symposium répondra à cette question.

FEINENDEGEN : What is the magnitude of the isotopic effect during the oxydation, after the catalytic reduction ?

WINAND : I have no idea about the isotopic effect.

INFLUENCE DE LA STRUCTURE
DES SOLUTIONS CONGELÉES DE THYMIDINE MARQUÉE
(^{3}H ET ^{14}C) SUR LEUR STABILITÉ

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On connaît bien maintenant l'autodécomposition des molécules organiques marquées par le ^{3}H et conservées en solutions aqueuses à 0 ou 4 °C. Ce phénomène général d'autodécomposition est particulièrement gênant pour l'expérimentateur qui est contraint d'utiliser ces solutions dès leur réception. Nous nous sommes proposé d'étudier ces phénomènes d'autodécomposition aux basses et très basses températures pour tenter de définir les meilleures conditions de conservation de ces solutions. La molécule choisie est la thymidine.

STABILITÉ DES SOLUTIONS DE THYMIDINE

A) AUTODÉCOMPOSITION DE THYMIDINE TRITIÉE

I. — TECHNIQUES ET MÉTHODES

Deux solutions de thymidine tritiée (^{3}H porté par le carbone 6), préparées par les services d'Amersham, nous ont été livrées dans la semaine même de leur préparation. Leurs caractéristiques sont résumées dans le Tableau I. La préparation

TABLEAU I. Caractéristiques des solutions de thymidine-^{3}H étudiées

La durée de l'expérience étant petite devant la période du ^{3}H (12,4 ans), nous n'avons pas tenu compte de la décroissance et avons supposé constante, durant toute l'expérience, l'énergie libérée par unité de volume et de temps.

Nᵒ des solutions	I	II	III
Activité spécifique (Ci/mM)	9	6,9	5,5
Energie libérée par ml de sol. 5.10⁻⁴ M (rads jours⁻¹)	1 314	1 020	807
Température d'étude (degrés centigrades)	0; —20; —75	0; —20; —196	—20 après congélation à —196

des expériences et les techniques utilisées au cours de cette étude étaient celles déjà décrites [1-3] (*).

II. — Résultats

Les courbes des figures 1 et 2 montrent que les deux solutions étudiées se décomposent de façon comparable : les résultats sont donc reproductibles. En présence ou absence de cystéamine, aux températures étudiées, *sauf —20 °C*, les pourcentages de thymidine décomposée sont des fonctions linéaires de la dose; c'est-à-dire que les vitesses d'autodécomposition sont des constantes qui dépendent des conditions expérimentales. Les vitesses d'autodécomposition de la thymidine sont toujours diminuées en présence de cystéamine; elles sont du même ordre de grandeur à 0 et —75 °C, mais plus lentes à —196 °C.
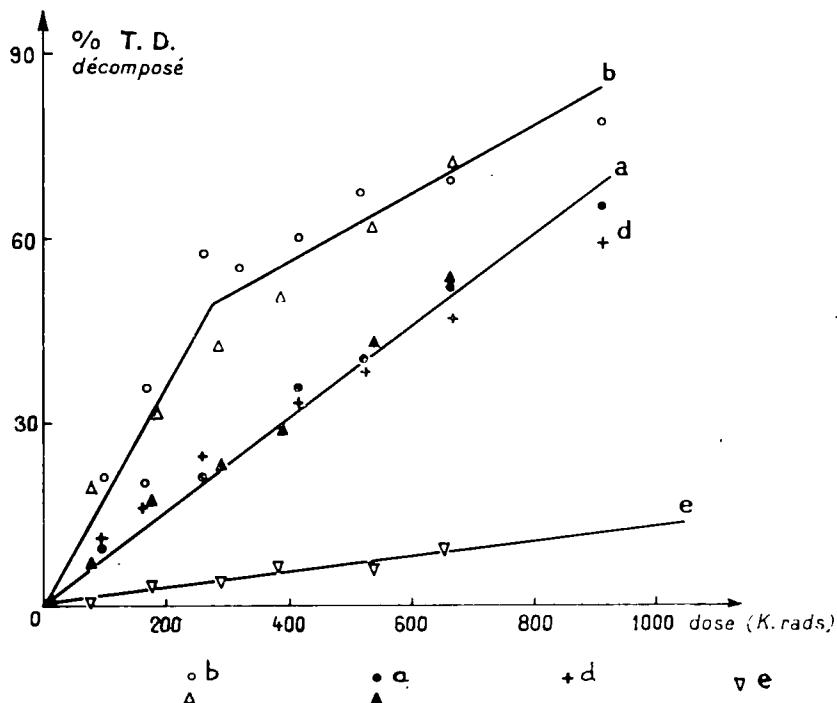
FIG. 1. Evolution des solutions aqueuses de thymidine tritiée conservées en l'absence de cystéamine.
a) 0 °C;   *b*) —20 °C;   *d*) —75 °C;   *e*) —196 °C.
o + ●   solution I;   △ ▲ ▽   solution II.

(*) Comme précédemment [2] nous désignons les composés non identifiés par « *y* »; la thymidine par T. D.; la thymine par thy; les composés d'oxydation (peroxydes cis et trans, glycols cis et trans) par P. O. La solution II contenait une molécule radioactive non identifiée que nous désignons par « *z* »; elle représentait 6 % de la radioactivité de la solution et semble s'être conservée intacte au cours de l'expérience.

A 20 °C, l'autodécomposition de la thymidine tritiée est représentée par une courbe cassée (fig. 1 et 2) qui définit deux vitesses. L'autodécomposition initiale se caractérise par une vitesse élevée, supérieure même à celle observée à 0 °C. Lorsque, en présence de cystéamine, 35 % de thymidine tritiée, et en l'absence de cystéamine, 50 % de thymidine tritiée ont été décomposées, les vitesses diminuent et deviennent respectivement 55 et 75 % de celles observées à 0 °C.
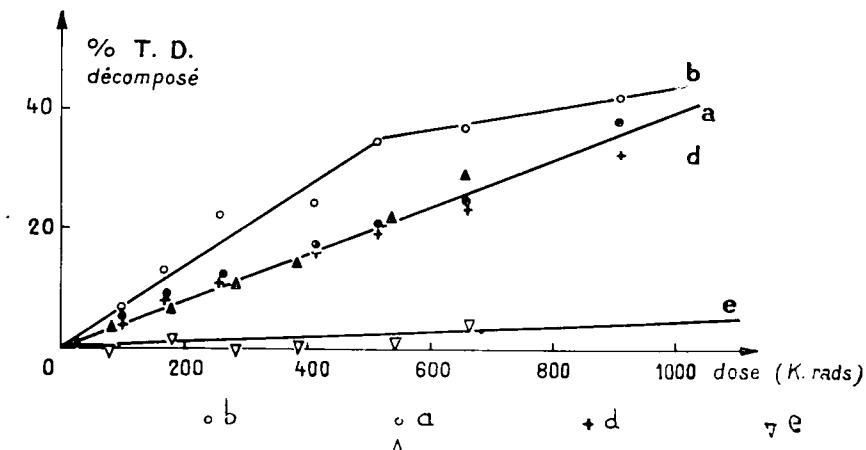

FIG. 2. Evolution des solutions aqueuses de thymidine tritiée conservées en présence de cystéamine. a) 0 °C; b) —20 °C; d) —75 °C; e) —196 °C.

o + ● solution I; ▼ △ solution II.

La figure 3 montre l'évolution de la solution II conservée à —196 °C ou à —20 °C, pendant le même temps, et en l'absence de cystéamine. Elle montre claire-ment la différence des vitesses de décomposition à ces deux températures.

B) RADIOLYSE DE THYMIDINE « FROIDE » OU TRITIÉE

Comme nous l'avons déjà souligné [1-3], l'autodécomposition observée est une radiolyse liée à celle de l'eau.

Les expériences antérieures [1] avaient établi une corrélation satisfaisante entre l'autodécomposition de thymidine tritiée conservée en solution aqueuse à 4 °C et la radiolyse par rayons X, à 0 °C, de thymidine froide en solution aqueuse (rayons X provenant d'un tube Machlett AEG 50 fonctionnant sous 40 kV).

Cette comparaison a été poursuivie à —75 °C, et les résultats obtenus sont résumés dans le tableau II. Ils montrent que, à cette température, en solution aqueuse et à dose comparable, ni la thymidine froide, ni la thymidine tritiée ne sont décom-posées par les rayons X ou les β du tritium porté par les molécules d'eau. Ces résultats sont différents de ceux observés sur les solutions à 0 °C [1] ou sur la thy-midine-³H à l'état sec à —196 °C [4]; dans ce cas, les auteurs ont pu montrer que
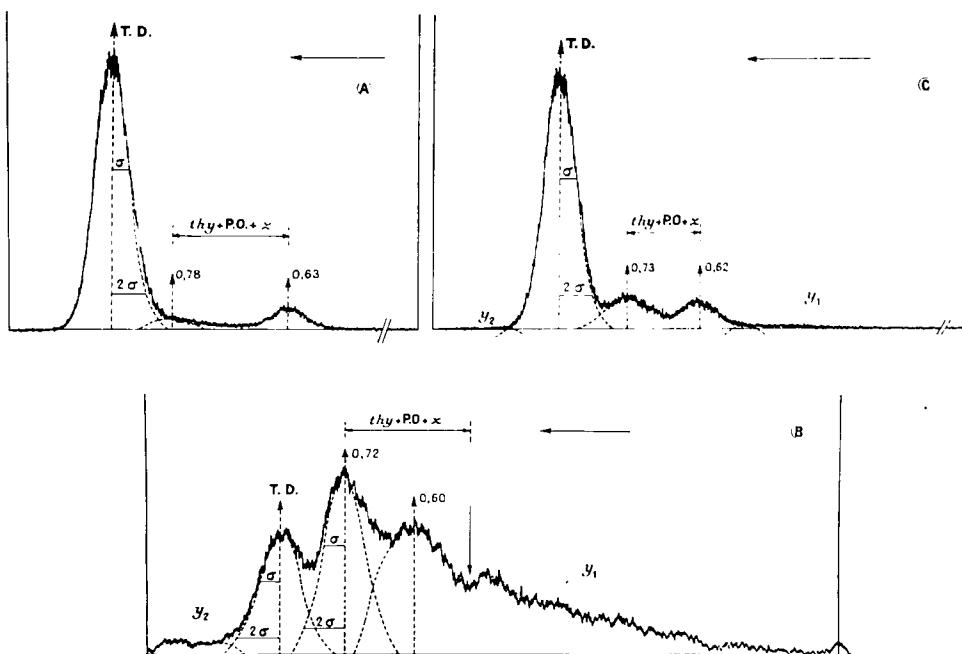
FIG. 3. Solution II conservée en l'absence de cystéamine : enregistrement de la radioactivité des chromatogrammes.

Chromatographie utilisant l'isopropanol-HCl comme solvant.

Courbe A : Analyse faite à $t = 0$ jour;

Courbe B : Analyse faite à $t = 644$ jours (656 000 rads) solution conservée à —20 °C.

L'échelle des ordonnées est multipliée par 10/3;

Courbe C : Analyse faite à $t = 644$ jours (656 000 rads) solution conservée à —196 °C.

Courbes	Rf (T. D.)	Rf (thy + glycols cis)	Rf (glycols trans + peroxydes + z)
A	0,86	0,78	de 0,78 à 0,63
B	0,81	0,72	de 0,72 à 0,60
C	0,82	0,73	de 0,73 à 0,62

TABLEAU II. Autodécomposition et radiolyse de thymidine froide ou tritiée

Concentration des solutions : 5.10^{-4} M; température : —75 °C

Source	Débit (rads. jours⁻¹)	Dose (rads)	% décomposé	
			T. D.	T. D. ³H
T. D. ³H	1 314 pendant 230 j	302 000		22 % [a]
Rayons X	Dose unique 1 200 pendant 250 j Dose unique	300 000 300 000 3 000 000	0 0 14	0
Eau tritiée 40 mCi/ml	11 500 pendant 30 j	345 000	0	0 [b]

[a] Cette expérience est faite avec la solution I, tableau I; les 22 % de thymidine décomposée sont déduits de la courbe *d* (fig. 1).

[b] Dans cette expérience, la thymidine tritiée utilisée avait une activité spécifique de 2,7 Ci/mM et une pureté radiochimique, vérifiée au laboratoire, de 95 %. Par suite de sa propre radioactivité, la thymidine tritiée utilisée dans ces expériences s'auto-irradiait à raison de 400 rads. jours⁻¹. Cette auto-irradiation était négligeable devant l'iradiation provoquée par l'eau tritiée : 11 500 rads. jours⁻¹.

l'autodécomposition de thymidine-³H évolue qualitativement et quantitativement comme sa décomposition par rayons X ou γ.

C) THYMIDINE-¹⁴C

Nous avons analysé une solution de thymidine-¹⁴C (¹⁴C porté par le carbone 2) conservée 930 jours au laboratoire à —75 °C. Cette solution se caractérisait par :

Activité spécifique : 30,7 mCi/mM; concentration de la solution : $2,6.10^{-3}$ M; énergie dissipée dans la solution en 930 jours : 160 000 rads. L'analyse faite n'a pas décelé la présence de produits d'autodécomposition. A la même dose, la thymidine tritiée en solution aqueuse et à —75 °C aurait été décomposée à 12 % (fig. 1).

L'ensemble des résultats précédents peut se résumer de façon simple en disant que, à basse température, la thymidine tritiée, et elle seule, *n'est sensible qu'aux* β *de ses propres atomes de* ³H.

D) DISCUSSION

Des résultats analogues ont déjà été rapportés par Evans et Stanford [5-6] qui ont signalé : 1° la stabilité de la thymidine-¹⁴C comparée à la radiosensibilité de la thymidine-³H lorsque leurs solutions aqueuses sont conservées à basse température, et 2° une vitesse d'autodécomposition plus élevée pour les solutions de thymidine-³H conservées à —20 °C. Nos résultats montrent également une radiosensibilité accrue de la thymidine tritiée en solution aqueuse conservée à —20 °C mais une radiosensibilité égale à —75 °C et 0 °C.

L'interprétation de ces faits pose de multiples problèmes. D'une part, il y a divergence entre résultats d'autodécomposition et d'irradiation par rayons X uniquement pour les solutions aqueuses congelées (—75 °C); d'autre part, il n'est pas usuel de constater une augmentation ou une absence de variation des vitesses de réactions lorsque la température diminue. Des phénomènes comparables ont été signalés en 1957 [7] dans l'étude de la radiolyse de dérivés iodés, sans toutefois avoir été interprétés.

L'ensemble de ces résultats suggère deux hypothèses :

— ou en solution aqueuse, les molécules de thymidine sont distribuées non pas au hasard, mais groupées en amas;

— ou dans les seules solutions congelées, la répartition des molécules du soluté est hétérogène.

L'une ou l'autre de ces hypothèses implique qu'il faut reprendre le calcul des doses. Il est évident, en effet, que si les molécules radioactives sont groupées, l'énergie des particules β du ³H se trouve concentrée localement au voisinage des molécules elles-mêmes par suite du faible parcours moyen des particules β du tritium (1 μ dans l'eau).

Les résultats négatifs obtenus par le Professeur Tonnelat et ses collaborateurs dans des expériences de diffusion latérale ou à angle nul de la lumière par les solutions concentrées de thymidine n'ont pas permis de retenir l'hypothèse de l'existence, en quantité notable, de groupements micellaires (*). Restait l'hypothèse de l'hétérogénéité des solutions congelées.

STRUCTURE DES SOLUTIONS CONGELÉES DE THYMIDINE

L'étude des réactions photochimiques de solutions congelées contenant des dérivés pyrimidiques [8]; celle de la radiolyse de différents composés soufrés en solutions aqueuses congelées [9], ont amené leurs auteurs à supposer que, en solutions congelées, les molécules de soluté sont à l'état d'agrégats. En 1961, Wang [8] a pu mettre en évidence l'hétérogénéité de solutions congelées. Pour interpréter les résultats décrits dans la première partie, nous nous sommes proposé de reprendre l'étude de la structure des solutions congelées en utilisant la thymidine tritiée des expériences précédentes. Les résultats détaillés ont fait l'objet d'une publication [10].

MESURES D'ACTIVITÉ

Partant d'une solution-mère à 5.10^{-4} M, nous avons préparé les solutions suivantes (eau monodistillée) :

(*) Nous remercions vivement le Professeur Tonnelat et ses collaborateurs qui ont accepté, à notre demande, de réaliser ces expériences.

Solution I   : 5.10^{-4} M   —4,62 mCi/mM
Solution II   : 5.10^{-3} M   —0,462 mCi/mM
Solution III : $2,5.10^{-4}$ M —4,62 mCi/mM
Solution IV : 5.10^{-4} M   —4,62 mCi/mM ⎧ dans un mélange eau-glycérine
Solution V : 5.10^{-5} M   —46,2 mCi/mM ⎰ à 10 % de glycérine.

Les mesures de radioactivité étaient faites avec un spectromètre à fonctionnement automatique de Nuclear Enterprises donnant dans nos conditions de mesures, une efficacité de comptage de 17 % pour les β de ^{3}H, avec un mouvement propre de 55 coups/minute.

A) 1re SÉRIE D'EXPÉRIENCES

I. — TECHNIQUES

On congelait 0,5 ml de solution dans le fond hémisphérique d'un tube en plastique de 12 mm de diamètre intérieur. Deux techniques étaient utilisées :

a) *Congélation autour d'un support et décongélation par couches concentriques*

Une tige de verre effilée (diamètre 0,9 mm) terminée par une boule était maintenue au centre de la solution. Après congélation, le tube était réchauffé à la main. La décongélation étant amorcée, la masse solide était extraite et fixée par la tige de verre à un support. On laissait la décongélation se faire librement et on recueillait successivement sur des verres de montre les gouttes formées (5 à 50 μl). Nous avons vérifié que la présence de la tige ne modifiait pas les phénomènes.

b) *Congélation sans support et décongélation par plans*

La congélation ayant été obtenue comme ci-dessus, sans support, et la décongélation étant amorcée, on pouvait faire adhérer la face supérieure du glaçon à un agitateur préalablement refroidi. On décongelait alors en appliquant successivement la partie inférieure en différents points d'une boîte de Pétri tiède. La décongélation avait lieu ainsi par plans successifs.

II. — RÉSULTATS

Trois températures de congélation ont été utilisées : —20 °C, —75 °C et —196 °C.

1° *Solutions aqueuses I, II, III*

a) *Congélation à —196 °C*

La congélation complète se faisait en 15 secondes. Elle débutait au fond du tube, se propageait rapidement à partir des parois, puis la surface libre gelait, emprisonnant une goutte qui cristallisait en dernier. La figure 4 montre la répartition des molécules de thymidine tritiée pour une solution 5.10^{-4} M. Les résultats sont peu différents pour une concentration de $2,5.10^{-4}$ M. L'hétérogénéité est accentuée à 5.10^{-3} M.
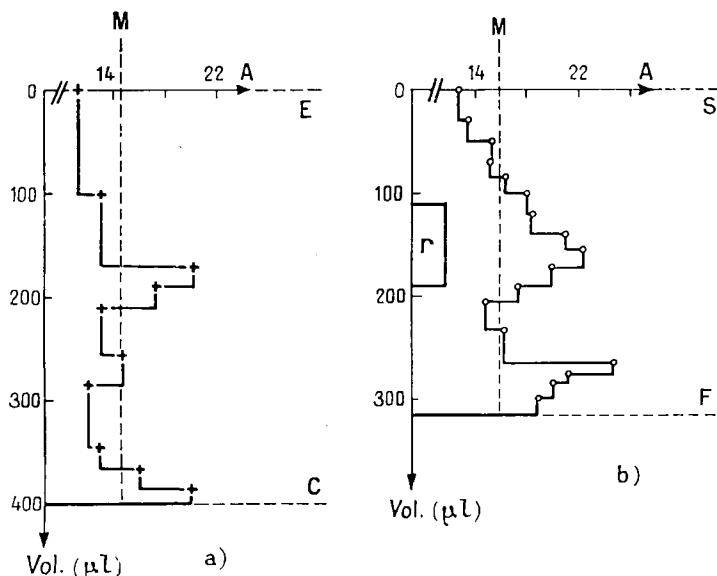
FIG. 4. —196 °C. Solution aqueuse I (5.10⁻⁴ M).

a) technique a (*);
b) technique b.

(*) Les lettres utilisées dans les figures 4 à 7 ont les significations suivantes :
A = activité de 20 μl en 10^3 coups/min; E = couche superficielle; C = cœur de la masse gelée;
S = surface libre; F = fond de la masse gelée; M = activité moyenne; r = volume et activité
de solution restée au fond du tube et provenant de la couronne la plus superficielle.

b) Congélation à —75 °C

La congélation se faisait en 5 minutes. Elle débutait au fond et se propageait par couches superposées. La moitié environ de la solution étant gelée, la surface cristallisait à son tour et enfin la couche sous-jacente. Pour une solution à 5.10^{-4} M, la répartition des molécules du soluté est hétérogène (fig. 5). La zone la plus concentrée est sous la surface supérieure. Elle ne contient cependant que 20 % environ des molécules. Cet excès est compensé par une faible concentration de la zone extérieure. Dans le reste du volume, la concentration est voisine de celle de la solution initiale. Cette hétérogénéité est plus accentuée dans les solutions à 5.10^{-3} M et très atténuée à $2,5.10^{-4}$ M.

c) Congélation à —20 °C

Les solutions passaient de la température ambiante à celle de —20 °C d'un congélateur. Dans ces conditions, le temps de refroidissement de la solution avant le commencement de la congélation était grand vis-à-vis de la durée de celle-ci. Le temps total était de 30 minutes. La congélation commençait au fond du tube et se propageait à partir des parois. La surface libre du liquide gelait ensuite, puis l'ensemble de la solution cristallisait rapidement.

Dans ces conditions, on observait une hétérogénéité importante (fig. 6).
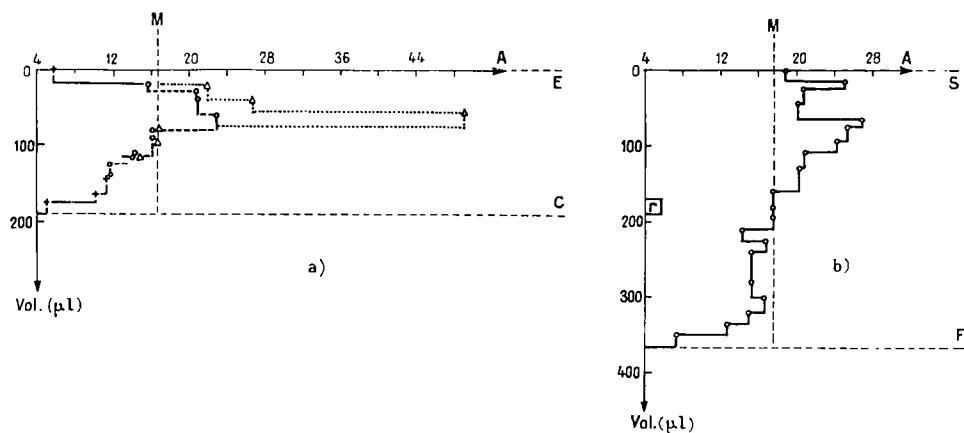
FIG. 5. —75 °C. Solution aqueuse I (5.10⁻⁴ M).

a) Technique *a* :

o : liquide provenant des seules couches inférieures;

△ : liquide provenant des seules couches supérieures;

+ : liquide provenant du mélange des couches supérieures et inférieures.
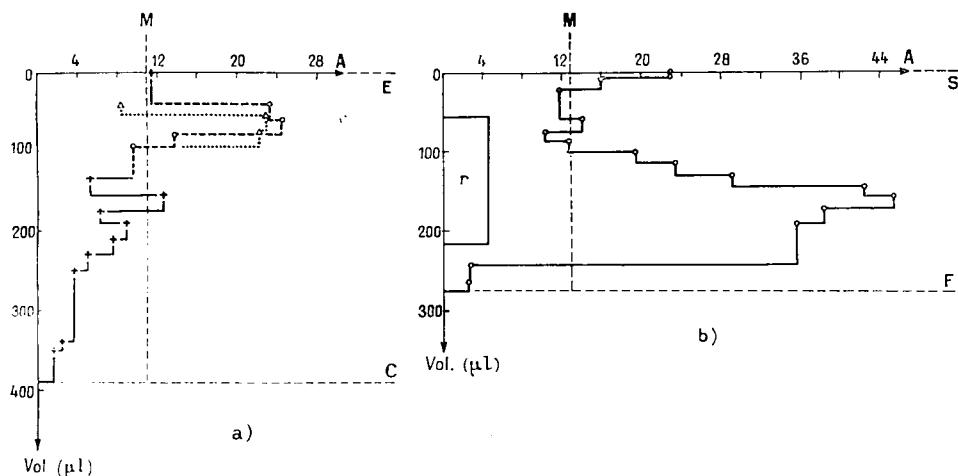
b) technique *b*.


FIG. 6. —20 °C. Solution aqueuse I (5.10⁻⁴ M).

a) technique *a* :

o : liquide provenant des seules couches inférieures;

△ : liquide provenant des seules couches supérieures;

+ : liquide provenant du mélange des couches supérieures et inférieures.

b) technique *b*.

La zone la plus concentrée renferme environ 50 % des molécules de thymidine. L'hétérogénéité est du même ordre lorsque la concentration est plus faible; elle est nettement atténuée lorsque la concentration est plus grande.

2° *Solution IV à 10 % de glycérine*

Le processus de congélation d'une solution glycérinée est différent de celui d'une solution aqueuse. Lorsque le tube est refroidi, un premier cristal de glace apparaît dans le fond puis une multitude de petits cristaux qui envahissent la solution.

La répartition des molécules de thymidine est très différente de celle observée dans une solution aqueuse. A —196 °C, toute hétérogénéité disparaît (fig. 7a). A —75 °C, l'hétérogénéité est faible; la zone la plus concentrée est située à la partie inférieure (fig. 7b), alors qu'elle était près de la surface supérieure dans les solutions aqueuses (fig. 5b). A —20 °C, une importante hétérogénéité subsiste (fig. 7c), elle est toutefois très inférieure à celle observée dans les solutions aqueuses. Le rapport des activités extrêmes passe, en effet, de 19 (fig. 6b) à 2,8 (fig. 7c).
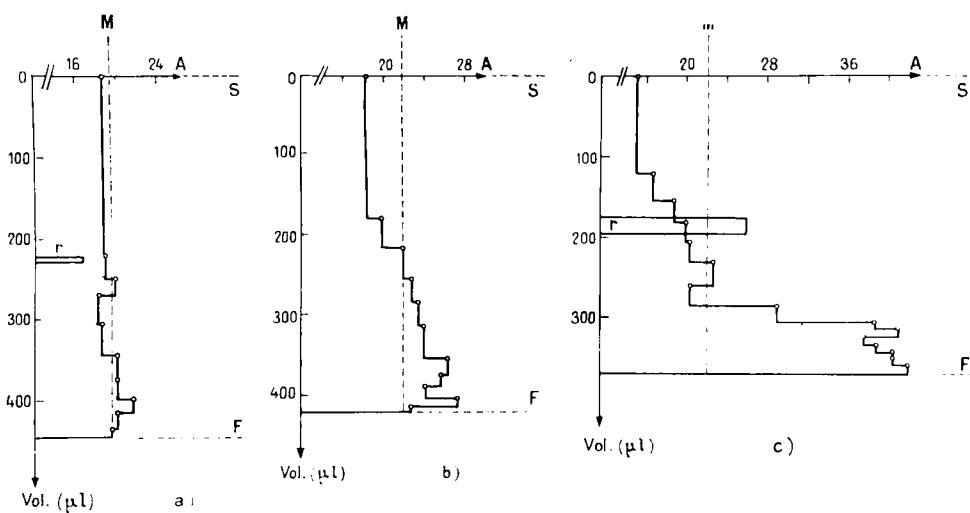
FIG. 7. Solution IV à 10 % de glycérine (5.10^{-1} M en T.D.); technique *b*.

a) —196 °C;
b) — 75 °C;   dans cette expérience *r* = 0.
c) — 20 °C.

B) 2ᵉ SÉRIE D'EXPÉRIENCES

I. — TECHNIQUES

Une des solutions I, II ou III était introduite dans des tubes de pyrex de différentes dimensions :

a — diamètre intérieur 3,4 mm, longueur 100 à 500 mm;
b — diamètre intérieur 9,8 mm, longueur 100 mm.

Les tubes étaient fixés à un support à crémaillère et placés au-dessus d'un Dewar contenant, soit de l'azote liquide, soit de la neige carbonique. Les tubes étaient descendus progressivement jusqu'à ce que le volume de liquide surnageant soit réduit à environ 50 μl qui étaient prélevés et dosés.

II. — Résultats

Quelles que soient les solutions, la température et la vitesse de congélation· l'excès de concentration en thymidine du surnageant n'a jamais atteint une valeur significative. Ces résultats montrent qu'il n'y a pas de transfert *progressif* entre la glace et le surnageant. Les conditions d'expérience ne permettent pas d'observer l'hétérogénéité de la glace.

C) 3e SÉRIE D'EXPÉRIENCES

I. — Techniques

Nous utilisions les solutions IV et V à 10 % de glycérine dans des tubes d'une ultra-centrifugeuse Spinco. Les tubes, remplis et fermés, étaient plongés dans un bain à —15 °C. Dès l'apparition des premiers cristaux de glace, ces tubes étaient placés dans le rotor, préalablement refroidi à —25 °C. On centrifugeait pendant 20 minutes, la température du rotor étant maintenue vers —17 °C.

Après centrifugation, on vidait les tubes dans un entonnoir refroidi à 0 °C au-dessus d'un tube à essais. On séparait ainsi facilement le glaçon du liquide. On laissait fondre la surface du glaçon avant d'en prélever différents échantillons pour en doser l'activité.

II. — Résultats

Le glaçon obtenu, exempt de glycérine, est constitué par l'agglomération, sous l'effet de la centrifugation, des petits cristaux formés en divers points de la solution.

TABLEAU III. Répartition de la thymidine-^{3}H dans un glaçon
(Etude faite avec la solution IV (5.10^{-4} M; 10 % glycérine) - Centrifugation à 68 000 g)

		Volume (μl)	Activité (coups/min)	Activité rapportée à 20 μl (coups/min)
Solution avant congélation		20		22 800
Glaçon	Fraction supérieure	15	5 894 •	7 850
	Fraction inférieure	16	6 920	8 650
	Centre	20 10	7 690 3 580	7 420

On constate que la répartition du soluté dans le glaçon est homogène (Tableau III) et qu'elle est une fonction décroissante de la vitesse de centrifugation. Lorsque celle-ci augmente, la concentration diminue, à partir de la valeur observée au repos, jusqu'à une limite inférieure atteinte vers 68 000 g.

D) Discussion

Les expériences d'ultra-centrifugation nous ont amenés à supposer que les molécules du soluté sont fixées dans la glace selon deux processus différents. On trouve en effet dans la glace centrifugée à très grande vitesse une concentration proportionnelle à celle de la solution initiale (environ 30 %). Cette limite inférieure de l'activité de la glace est atteinte vers 68 000 g et doit correspondre aux molécules de soluté incorporées dans les cristaux pendant leur formation, la durée de leur séjour dans la solution étant très courte. Lorsque l'accélération diminue, de 68 000 g au repos, ce temps de séjour augmente et la quantité croissante de soluté mesurée dans la glace s'y trouve fixée par adsorption à la surface des cristaux. La concentration maximum atteinte au repos, correspondrait à une saturation dont le taux dépend des conditions d'expérience.

La formation, par adsorption, d'amas de molécules de thymidine dans les cristaux (hétérogénéité moléculaire) entraîne des variations de concentration de la solution pendant que la cristallisation progresse. Il en résulte l'hétérogénéité macroscopique observée dans la 1re série d'expériences. Dans la 2e série, les molécules du soluté sont incorporées dans la glace au fur et à mesure de sa formation. Une analyse très fine de la structure du glaçon ferait peut-être apparaître une hétérogénéité.

L'hétérogénéité macroscopique dépend des conditions dans lesquelles se développe la congélation : température, concentration, solutions aqueuses ou glycérinées, forme du récipient, etc. La température détermine la vitesse de congélation, les phénomènes de saturation sont liés à la concentration, d'autres conditions d'expérience influent sur la forme des cristaux de glace et la progression de leur développement.

DISCUSSION ET INTERPRÉTATION DES RÉSULTATS

Les expériences décrites dans la deuxième partie nous amènent à supposer que si, dans une solution congelée, les molécules de soluté ne syncristallisent pas avec les molécules d'eau, les premières ne sont que pour une faible partie incorporées dans les cristaux de glace au cours de leur formation. *Le processus dominant est l'adsorption* à la surface de ces cristaux, adsorption localisée en des points particuliers selon le mode de croissance des cristaux. Il en résulte la formation d'amas créant une hétérogénéité à l'échelle moléculaire qui peut avoir pour conséquence une hétérogénéité macroscopique.

L'hétérogénéité à l'échelle moléculaire qui existe à chaque température de congélation démontre que, pour une solution congelée, *le calcul de la dose E fait en supposant une répartition homogène des molécules de thymidine-³H dans la masse solide, est faux.* Soit E_1 l'énergie en rads libérée au voisinage des molécules de soluté dans une solution congelée. La comparaison radiolyse par rayons X et auto-décomposition (Tableau II) indique que le même pourcentage de thymidine décomposée est obtenu lorsque E_1/E est voisin de 15.

Cette hétérogénéité à l'échelle moléculaire rend compte également de la stabilité de la thymidine-¹⁴C conservée en solution congelée.

CAS PARTICULIER DE —20 °C

L'hétérogénéité macroscopique, importante uniquement pour les solutions aqueuses congelées à —20 °C, explique que, à cette température, la vitesse d'auto-décomposition est supérieure à celle observée à 0 °C. En effet, à —20 °C, la congélation crée des zones où la concentration en soluté est deux à trois fois supérieure à celle résultant d'une répartition uniforme, alors que la vitesse de diffusion des radicaux formés dans l'eau est encore très importante. En modifiant cette hétérogénéité macroscopique par congélation préalable à —196 °C, on devait diminuer la vitesse d'autodécomposition. L'expérience l'a confirmé.

CONCLUSIONS

Les solutions aqueuses de thymidine tritiée conservées à basse température, subissent une autodécomposition faible à —196 °C, mais importante à —20° et —75 °C; en présence de cystéamine, l'autodécomposition est pratiquement nulle à —196 °C. Pour ces solutions aqueuses, l'autodécomposition observée à 0 °C est analogue à une radiolyse par rayons X; il en est de même pour la thymidine-³H conservée à l'état sec [4]; mais pour les solutions aqueuses congelées, les résultats sont différents.

Les solutions aqueuses de thymidine-³H peuvent être conservées longtemps à —196 °C et peu de temps à 0 °C, mais jamais à —20 °C.

Comme le montre la différence de comportement des thymidine-³H et thymidine-¹⁴C, la radiosensibilité de la première ne résulte pas de particularités de la thymidine mais du tritium. Elle est la conséquence de la faible énergie moyenne du rayonnement β du ³H (qui entraîne un faible parcours moyen dans l'eau) en liaison avec la structure particulière des solutions aqueuses congelées. On doit s'attendre à la retrouver pour l'une quelconque des molécules tritiées qui ne syncristallise pas avec les molécules d'eau.

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DISCUSSION

OSINSKI : Je voudrais savoir quelle était l'énergie des rayons X utilisés.

APELGOT : Ce sont des rayons mous. L'énergie du photoélectron était trois fois plus grande que celle du β du tritium.

OSINSKI : Nous étudions également les phénomènes de décomposition par auto-irradiation de stéroïdes tritiés. Bien qu'il soit difficile de comparer les stéroïdes et la thymidine, j'aimerais signaler que, dans une solution aqueuse apparemment homogène, à température ordinaire, nous avons dû utiliser des doses de 10^7 rads de rayons γ (Co) pour atteindre l'effet obtenu par 10^3 rads de rayons β. C'est donc un facteur plus important que dans vos expériences.

Bien que nous ne l'ayons pas encore vérifié, il n'est pas exclu que les stéroïdes, comme le cholestérol, puissent former des amas moléculaires. La concentration est très basse, mais il est possible que les molécules se trouvent à l'état de cristaux liquides donnant donc lieu à des concentrations locales plus élevées.

Je voudrais encore vous demander dans quel type de verre vous avez conservé la thymidine tritiée.

APELGOT : Du pyrex, donc du verre dur.

OSINSKI : Nous avons constaté que le phénomène ne se passe pas du tout de la même façon dans une ampoule en verre ordinaire et dans une ampoule en pyrex, dans le cas où le rapport volume/surface est assez désavantageux, c'est-à-dire plutôt dans des tubes que dans des ampoules.

ALTMAN : It is very well known from number of investigations that the molecules in a solid-liquid system when adsorbed on the solid phase receive a certain amount of radioprotection, in other words, that their destruction by radicals produced by radiant energy is lessened.

Now I wonder if a similar situation is obtained in your studies where you seem to demonstrate that there is an adsorption of thymidine on a crystal surface. Does this alter the susceptibility of a molecule to destruction in a similar way ?

APELGOT : Les résultats montrent que c'est le contraire. Beaucoup de gens ont montré que si l'on conserve certains types de molécules dans des solutions alcooliques, on observe une vitesse d'autodécomposition plus basse que quand on conserve dans une solution aqueuse et je pense que cela vient du fait que les solutions alcooliques ne congèlent pas de la même façon. Et ce phénomène d'adsorption n'est justement pas quelque chose d'uniforme à la surface du cristal ou de la phase solide.

PICHAT : Je voudrais savoir si vous avez travaillé sur des solutions stériles et, si oui, ces solutions étaient-elles sous vide ou en atmosphère d'azote ou inerte ?

APELGOT : Les solutions étaient stériles, mais conservées en atmosphère normale.

PICHAT : Nous avons nous-mêmes constaté que la conservation de la thymidine à —196 °C a un effet bénéfique; j'ai ici des exemples de radiochromatogrammes de thymidine conservés à —20 °C et à —196 °C pendant 6 mois. Il n'y a pas de comparaison. On observe à —20 °C peut-être 6 ou 7 produits, alors que, au bout de 6 mois dans l'azote liquide le produit est encore utilisable.

Pour ce qui a trait au clivage de votre cristal de glace, je crois savoir que les gens qui effectuent des dosages très précis de teneurs en deutérium des eaux naturelles (sur des grelons, par exemple), à Saclay, utilisent un microtome pour scier les cristaux. Est-ce que, dans votre cas, on ne pourrait pas le faire ?

APELGOT : Nous n'avions pas, à l'Institut du Radium, de microtomes adéquats. Mais les résultats étaient, malgré tout, suffisants pour montrer la différence qu'on pouvait obtenir par l'emploi de températures basses.

PICHAT : Quelle méthode analytique employez-vous dans les expériences avec la thymidine inactive (par exemple, les expériences avec rayons X) ?

APELGOT : On détecte alors la thymidine intacte par spectrophotométrie U.V., ce qui est relativement précis. A —75 °C, on n'a pas pu mettre en évidence d'auto-décomposition de la thymidine tritiée irradiée par rayons X. Les deux techniques ne donnent certainement pas les mêmes résultats. Je pense que les erreurs de l'une sont différentes des erreurs de l'autre, mais doivent être à peu près du même ordre de grandeur.

AMELINCKX : Je me demande dans quelle mesure l'origine d'inhomogénéité dans les solutions congelées ne doit pas être recherchée dans un phénomène de raffinage par zone fondue, qui est bien connu du physicien pour raffiner des substances cristallines.

APELGOT : Nous avons pensé à cela et c'est pourquoi nous avons fait l'expérience dans les tubes, parce que si le phénomène était celui-là, en faisant des congélations lentes dans les tubes, on aurait dû avoir une variation de la concentration dans les derniers surnageants, et cela nous ne l'avons jamais observé et je sais que d'autres gens, justement parce qu'ils soupçonnaient l'hétérogénéité des solutions congelées ont fait ces expériences et montré qu'il n'y avait pas de variations d'activité dans le dernier surnageant. Ce n'est donc pas exactement le même phénomène. Et je pense que tout se passe à l'intérieur de la partie de la solution qui est en train de congeler(*).

(*) Au cours d'expériences ultérieures, nous avons pu observer ce phénomène de « zone fondue ». Il est nécessaire d'utiliser une vitesse de congélation beaucoup plus lente que celle de nos premiers essais.

EVANS : Some of these results if not all the results could have been explained by the activation energy required for the reaction between the free radicals and the solute. At a low temperature the activation energy would favour radical-radical recombination rather than radical-solute inter-reaction. As you raise the temperature to above —143° C, radical solute inter-reaction becomes more important, and as the temperature raises further still the radical recombination reaction is favoured again at the high temperatures when you reach over 0° C.

APELGOT : C'est exact mais, dans ces conditions, la formation de radicaux est semblable dans le cas des β du tritium et dans le cas des photoélectrons des rayons X mous. On ne pourrait donc pas avoir un effet dans un cas et rien dans l'autre, s'il s'agissait de réactions radicalaires. Dans l'expérience faite avec de l'eau tritiée, que les rayons β viennent de l'eau tritiée ou qu'ils viennent de la thymidine, la réaction avec les radicaux est forcément la même. Or, avec des réactions radicalaires identiques, on observe cependant une autodécomposition dans un cas et on n'en observe pas dans l'autre.

DISTRIBUTION OF TRITIATED THYMIDINE
IN ANIMALS AS SHOWN
BY WATER-SOLUBLE AUTORADIOGRAPHY

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A new technique is described for studying the incorporation of labelled soluble compounds, or for work on unextracted materials [1, 2]. The method is very simple and reliable and immobilizes labelled materials; no solutions which might leach out soluble compounds are involved until after radiographic exposure is complete. Frozen sections are cut in a Pearse cryostat kept in the darkroom, and "picked up" on cold cover-slips coated with Kodak AR.10 stripping film. The sections are kept frozen until the completion of the exposure.

FREEZING

Tissues were frozen by immersion in iso-pentane cooled with liquid nitrogen (—196° C). Frozen tissues can be stored in air-tight glass specimen tubes surrounded by powdered dry ice (—78° C) for 1-2 days. Storage for longer periods can be achieved in a liquid nitrogen refrigerator. Fitzgerald [4] found that freezing smears in this way and subsequently dehydrating them by freeze substitution in absolute ethanol caused no detectable loss of ^{32}P to the processing fluids. Freezing at higher temperatures, i.e. with solid CO_2 by conduction, causes vacuolation in some tissues due to ice-crystal formation. The reduced speed of freezing allows more time for redistribution of soluble compounds.

COVER-SLIPS

Cover-slips are used in preference to microscope slides because frozen sections can be picked up more easily with the thin flexible covers from the concave microtome knife, and because the relative position of emulsion and tissue of the completed preparations becomes the same as that of the standard stripping film autoradiograph. This makes interpretation easier when the two types of preparations are compared. Size 2″ ⨯ 7/8″ (50 × 22 mm) No. 1 cover-slips are smeared on both sides with glycerine-albumen and covered with Kodak AR.10 stripping film, êmulsion upwards, i.e. the reverse of the normal stripping film technique (Fig. 1). The filmed cover-slips are dried in a stream of air at room temperature for 2-3 hours until completely dry. Coated cover-slips can be stored in light-tight boxes at 2 to 4° C and cooled to —5 to —10° C when required. During the whole process, the cover-slips are stored, exposed, processed and stained in specially constructed perspex racks [1]

to prevent breakage through unnecessary handling. Cover-slips are identified by marking the emulsion on the underside with a felt pen such as Gem Marker of Magic Marker. This marking has been found to withstand all the processing and staining solutions and is easily scraped away with the excess film after mounting.
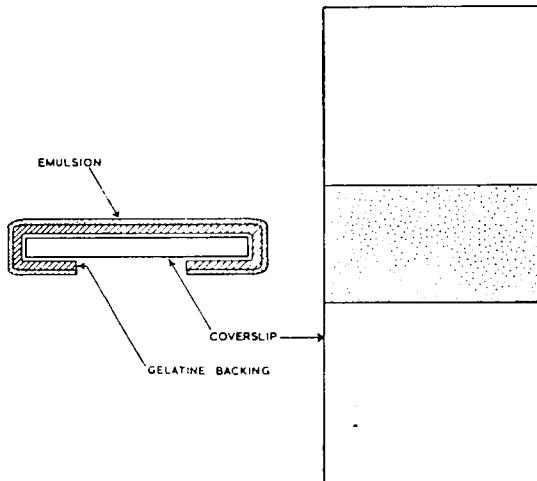

FIG. 1. Cover-slip covered with AR.10 stripping film, emulsion upwards. The gelatine backing of the film is in close apposition with the cover-slip. (Courtesy, *J. Roy. Micr. Soc.*)

SECTIONING

Sections 5-6 μ thick were cut in a Pearse cryostat regulated for —20 to —30° C and kept in the darkroom. Used and unused cover-slips are stored in light-tight plastic boxes. Attachment of tissue to block, trimming and any other adjustments are carried out in full light. When the material is sectioning satisfactorily the light is turned off, and sectioning continued under the red safe-light (Kodak Wratten Series 1). Immediately before use the filmed cover-slips are kept at —5 to —10° C. Sections are transferred by touching the cold cover-slips against the sections, as is usual with cryostat sectioning. The sections at cryostat temperature of —20 to —30° C stick securely to the film without melting and withstand subsequent processing and staining without movement. The knife is kept scrupulously clean and the safelight positioned so that its reflection is seen on the blade, sections then appear as black shapes and with practice their quality can be easily judged.

EXPOSURE

The completed preparations are exposed in light-tight plastic boxes with a dessicant (silica gel) either in the back of the cryostat, or at a temperature below —20° C. This prevents melting and eliminates possible enzyme action between

section and film. No other treatment is necessary. Freeze substitution in absolute ethanol at dry ice temperature and vacuum dehydration at low temperature showed no advantage over the simple technique of storing the autoradiographs at low temperatures. Low-temperature exposure did not seem to reduce appreciably the sensitivity of the film. The thin sections probably dry very rapidly even at these low temperatures.

PROCESSING

After suitable exposure the attached tissue sections were fixed in Wohlman's fixative (5 ml glacial acetic acid, 95 ml absolute ethanol) at 17° C for 1 min, and washed for 1 min in each of four changes of tap water (pH > 7.0). The section is on the outside of the film and therefore staining and fixing with solutions which would normally penetrate the film are feasible. Histological fixation is necessary because the developer, being strongly alkaline, otherwise causes gross disruption of the tissues as well as preventing the satisfactory uptake of stains. It is important that the autoradiographs should be carefully washed before development because the slightest trace of acetic acid will act as a photographic stop bath, and will retard or prevent development. Development was in Kodak D 19b developer at 17° C for 5 min and fixation in Johnson's Fixsol (diluted 1 : 10) for 12 min at 17° C. The section remains in secure contact throughout the processing.

STAINING

Since the section is on top of the film, the problem of stain penetration through the gelatine is eliminated and staining times are also greatly reduced. The staining techniques for Toluidine blue or Haematoxylin and Eosin are :

Toluidine blue 0.1 % aq.	10-39 seconds

(Toluidine blue should be checked before use. Some batches
act as reducer and so remove photographic grains.)

Ehrlich's haematoxylin diluted 1 : 3 with water	10-15 minutes
Wash in CO_2-free distilled water	30 seconds
Differentiate in 0.2 % HCl	15-30 seconds
Blue in running tap water	15-30 minutes
Eosin 1 % aq.	3-5 minutes
Differentiate in CO_2-free distilled water for	0.5-1 minute
	(by inspection).

After staining, the autoradiographs are air dried and the sections cleared with a drop of xylene. The cover-slip is inverted and mounted with a drop of Euparal on a standard glass slide (Fig. 2).

When the mountant has hardened, the excess film can be removed with a razor blade.
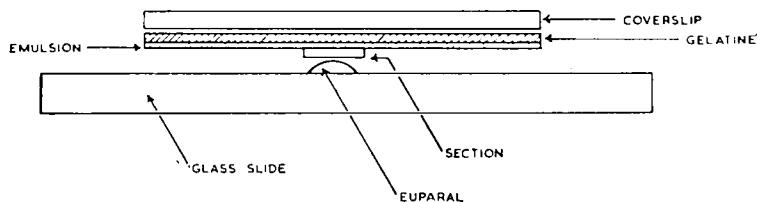
FIG. 2. The method of mounting the completed autoradiograph. (Courtesy, *J. Roy. Mic. Soc.*)

RESULTS

In one experiment adult mice weighing 22-25 g were given intraperitoneal injections of 50 µC tritiated thymidine and killed 15, 30 and 60 min after injection. After dissection, organs were immediately frozen by immersion in isopentane cooled in liquid nitrogen.

Autoradiographs of well-labelled nuclei were invariably above the nuclei, indicating that the film and tissue were firmly bonded together, and that no slipping occurred during processing or exposure. Autoradiographs of fixed and embedded material were also prepared.

Most cells in all tissues investigated show nuclear and cytoplasmic labelling 15 min after injection, while after 30 min the autoradiographs are considerably weaker. One hour after injection, the grain numbers are lower still, though definitely above background, and after 24 hr the counts are nearly equal to background except over cells which have incorporated tritiated thymidine into DNA. Such cells are found in varying proportions at all times in epithelial cells and smooth muscle surrounding the colon and seminal vesicle, and in brain, as well as in the dividing organs (intestine, oesophagus).
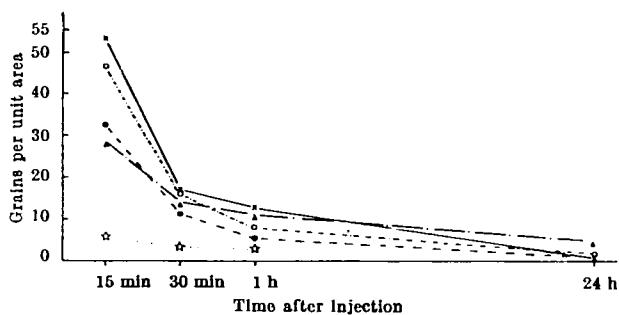
FIG. 3. Distribution of tritiated thymidine in various tissues of the mouse. The unit area for grain counting ($72 \mu^2$) was determined by an eye-piece aperture. Nuclei showing high labelling were assumed to have incorporated tritiated thymidine and were omitted. Crosses, smooth muscle (colon) triangles, colon; stars, brain; open circles, smooth muscle (seminal vesicle); closed circles, seminal vesicle. (Courtesy of *Nature.*)

The number of photographic grains per unit area was counted in various tissues by using a square eye-piece aperture. Heavily labelled nuclei were disregarded. Figure 3 shows that after intraperitoneal injection the concentration of grains is highest in smooth muscle, somewhat lower in epithelial tissues, and lowest in brain, probably owing to the blood-brain barrier.

In another experiment adult mice were given an intraperitoneal or intravenous injection of 50 μC tritiated thymidine and killed 2 min after injection; water-soluble autoradiographs were prepared as described. In the smooth muscle from the colon of a mouse which had been given an intravenous injection, the label is evenly distributed throughout the muscle. On the other hand, in smooth muscle (colon) from a mouse which had been given an intraperitoneal injection, thymidine can be seen diffusing in from the peritoneal fluid surrounding the intestine, irrespective of the direction of the muscle fibres or nuclear membranes. The speed of diffusion is approximately 2 μ/sec.

Grain counts show that after intravenous injection the concentration in mouse intestine is approximately equal in the epithelium and in the smooth muscle. Using an eye-piece aperture of apparent diameter equal to that of cell nuclei, the grain density over nuclei and cytoplasmic areas in brain was counted (Figs. 4, 5 and 6). Fifteen min after injection, the cytoplasm shows a reasonably random distribution of grain densities while some of the nuclei contain considerably more than others. After 30 min, a few nuclei show more grains than the equivalent areas of cytoplasm, while after 1 hr the distribution over nuclei and cytoplasm is almost equal. Similar differences between labelling of nuclei and cytoplasm were observed in other tissues, though no detailed counts were undertaken. In brain the average count per unit area over nuclei is 3.8 grains and 1.1 grains over cyto-
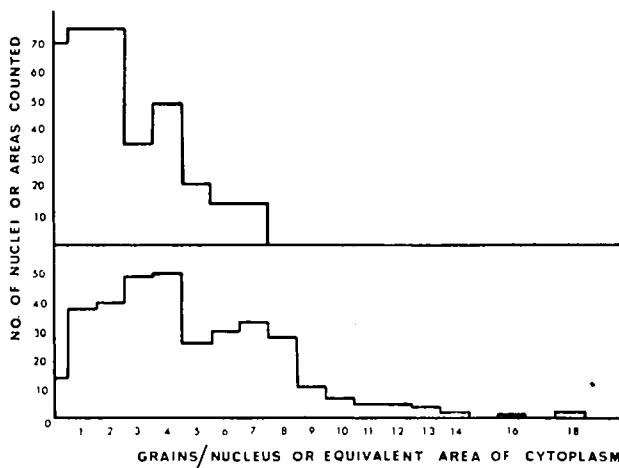
FIG. 4. Grain distribution in brain, 15 min after injection of tritiated thymidine. An eye-piece aperture of the apparent size of the nuclei was used to count grains over 300 nuclei and 300 cytoplasmic areas. Above, cytoplasm; below, nuclei. (Courtesy of *Nature*.)
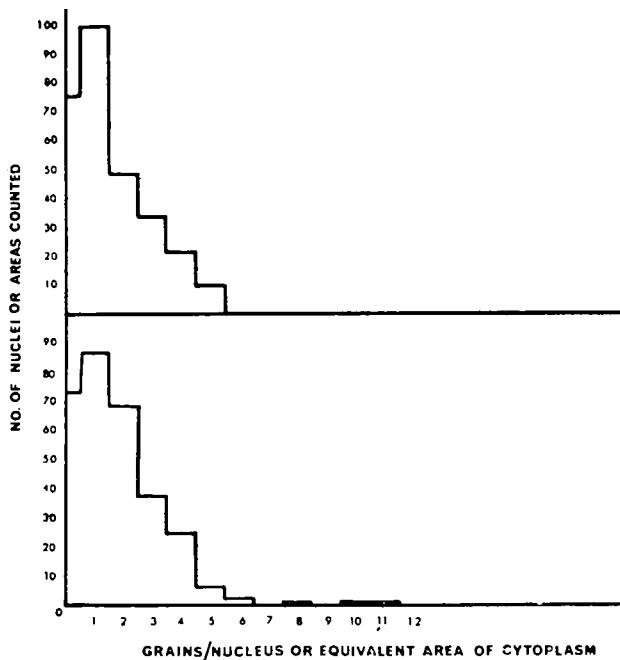
FIG. 5. Grain distribution in brain, 30 min after injection. See remarks to Fig. 4. Above, cyto-plasm; below, nuclei. (Courtesy of *Nature*.)
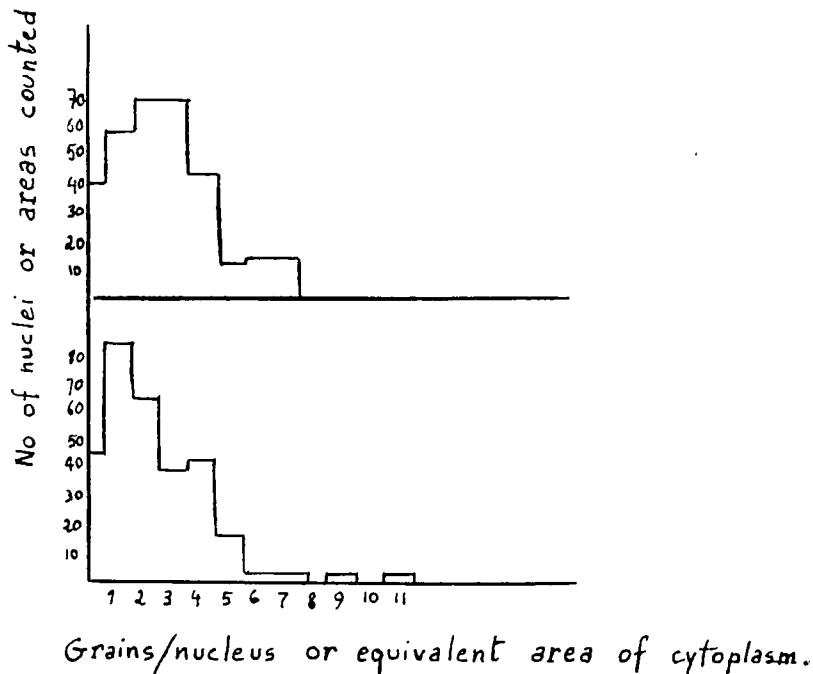
FIG. 6. Grain distribution in brain 1 hr after injection. See remarks to Fig. 4. Above, cytoplasm; below, nuclei. (Courtesy of *Nature*.)

plasmic areas. Measurements have shown that approximately 10 % of the area of a section is nuclear, hence 27.8 % of the labelled material is in the nuclei and 72.8 % in the cytoplasm. After 30 min the concentrations are 0.9 grains per unit area over nuclei and 0.7 over cytoplasm, and at 60 min 1.2 grains for nuclei and 1.6 for cytoplasm. These figures have been corrected for background. After 30 min and 60 min, the concentrations are approximately equal and therefore 10 % is in the nuclei and the remainder in the cytoplasm. Nevertheless a number of nuclei still show more grains than would be expected from a random distribution (Fig. 6). In previous work tritiated thymidine was found to be incorporated into DNA in only 0.24 % of the cells of the central nervous system; the labelled nuclei counted here in considerable numbers are therefore unlikely to be permanently labelled.

Grain counts over well-labelled nuclei in water-soluble autoradiographs and stripping film autoradiographs of fixed and embedded organs showed no significant difference. Feinendegen and Bond [3] found higher concentrations of tritiated thymidine in bone-marrow in cell types capable of division than in the non dividing ones. Unless the discrepancy between their findings and ours is due to differences in technique it has to be assumed that bone-marrow cells differ from those in other organs in permeability to thymidine.

Another series of experiments was performed on hair bulb [5]. If hair was plucked and thymidine injected afterwards, there was no label in the cells with normal techniques, 2 hr after injection. 16 to 19 hr after injection of tritiated thymidine, however, cells were labelled, after embedding. Therefore, those few cells must have obtained a supply of labelled thymidine which enabled them to make labelled DNA 16 hr later. We thought that those cells were originally dividing cells (matrix cells) and must have phosphorylating enzymes. They may have phosphorylated the thymidine; phosphorylated thymidine cannot diffuse out of the cell and remained in the cell until it starts synthezising DNA. By water-soluble autoradiography, we have been able to show that this is indeed the case : 2 hr after injection of thymidine, the cells are labelled [5].

It can be concluded that :

a) tritiated thymidine is available to the cytoplasm and nuclei of nearly all cells in the organs investigated;

b) since only a proportion of nuclei are found to be permanently labelled, availability of thymidine in itself is not sufficient to initiate incorporation of thymidine into DNA, nor does it to any extent stimulate it;

c) the amount of tritiated thymidine which diffuses into all cells in an organ is sufficient to explain the degree of uptake in well-labelled nuclei if it is assumed that such cells retain all the thymidine entering the cell and that the contents of soluble material are "turned over" once every 3-5 min;

d) the incorporation of tritiated thymidine in the more heavily labelled nuclei of the central nervous system in excess of that expected from random labelling may be due to a different permeability of some nuclear membranes or the formation of an intermediate compound;

e) in view of the short time of availability of tritiated thymidine, the site of injection can seriously affect the total amount of tritiated thymidine reaching the cells, and therefore comparison of grain numbers over labelled nuclei in different organs may be subject to substantial errors.

AKNOWLEDGMENTS

This communication is compiled from two papers : those of Appleton [1] and Pelc and Appleton [6]. We are grateful to the editors of these journals for permission to use this material

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DISCUSSION

HOLLDORF : I think that degradation of thymidine before utilization is a very important point. If we incubate *Coli* cells or ascites cells for 30 minutes with a given amount of thymidine, thymidine is split by phosphorylation to deoxy-ribose-1-phosphate and thymine. Thymine is quantitatively released into the medium and cannot be reutilized. Deoxyribose-1-P is converted to deoxyribose-5-P which is easily split into acetaldehyde and phosphoglyceraldehyde. For these reasons, at least in *Coli* and ascites cells, there is no more uptake of thymidine after 30 minutes incubation. With ascites cells, we compared the incorporation of ^{14}C-labelled C 1 compounds (formaldehyde or formate) and that of ^{3}H-thymidine into DNA. After degradation of the labelled DNA, we observed that the amount of TMP incorporated from thymidine is low compared to the extent of thymidine synthesis by the normal pathway (deoxyuridilic acid + C 1 compound). Only in the presence of inhibitors such as fluorodeoxyuridine or amethopterine can one significantly increase the amount of labelled thymidine incorporated.

PELC : A failure to incorporate has been noted before in *Coli* and I have forgotten to mention that bacteria of course frequently cannot utilize thymidine. We have grown HeLa cells and amnion cells and we did not notice any loss of thymidine, I mean a breakdown of thymidine in the culture medium.

We did it for up to 72 hours and counted the number of labelled cells and this increased in a perfectly linear fashion.

HOLLDORF : I agree with you, but in ascites cells, we also observed that thymidine phosphorylase is ten times more active than thymidine phosphokinase.

APELGOT : J'ai fait la même observation avec des pneumocoques et certaines souches d'*E. Coli* : quand on les fait croître en présence de thymidine marquée au ^{14}C ou au ^{3}H, au bout de quelque temps on retrouve de la thymine marquée dans le milieu, et l'incorporation dans les cellules est très faible.

NIGON : I can agree still more strongly with cells of *Bombyx Mori*. In half an hour, the whole thymidine which was in the medium is converted to thymine and degradation products of thymine. Thymidine phosphate also goes out of the cell.

PELC : But in a mammal, the breakdown of thymidine takes place in the liver.

NIGON : The difficulty with autoradiographic methods is certainly to make a distinction between thymidine, TMP, thymine, etc.

PELC : With water-soluble techniques, we can design extraction procedures. We have not done it yet, but instead of mounting the frozen section on the film directly, we can mount it on a microscope slide first, extract TMP by trichloracetic acid and then make a conventional stripping film autoradiograph.

SMITH : Did I gather from the graphs that the uptake of tritiated thymidine in muscle tissues was the same as the uptake in active gut cells ? If this is the case, how then do you postulate phosphorylating enzyme in this instance ?

PELC : There is more phosphorylating enzymes in the gut cells than in the muscle cells. In our autoradiographs most of the grains are due to unchanged thymidine.

FEINENDEGEN : I have a few technical questions and a few non technical remarks. There is a notorious difficulty in approximating a section, a frozen dry section to a dry nuclear track emulsion. How did you get it so close that you get those beautiful pictures ?

PELC : Because the section is not dry. Immediately after cutting, the frozen section is put on the film. A temperature gradient will help. If the film is slightly warmer than the section, then the section will stick.

FEINENDEGEN : Then you have a very tiny amount of moisture.

PELC : It is frozen all the time.

FEINENDEGEN : Do you leave this slide in the cryostat for a long time, in order to let it equilibrate or do you put the slide right on to the section ?

PELC : The slides are kept at —5 to —10° C and the tissue blocks at —25 to —30° C. After putting the sections on the slides, the preparations are kept in a refrigerator at —25° C during the exposure. Between +4 and —25° C, we loose approximately 20 % of the sensitivity of the photographic emulsion.

FEINENDEGEN : Do you have any idea to what degree you control your chemographic effect ?

PELC : We do not get any chemographic effect. I think the explanation is that most chemographs are due to enzyme action of unfixed cells, but at —25° C, the enzymes are inactive.

FEINENDEGEN : I have more remarks concerning the question whether thymidine is a natural precursor or not. This question is important for judging the function of thymidine kinase in the cells. I think there is a good chance that thymidine is a natural precursor for DNA. There is a considerable amount of DNA catabolites circulating in the peripheral blood, perhaps also in the form of thymidine. It may need the kinase in order to be incorporated again into DNA. I feel that the phosphorylating enzymes are present or active only in those cells which are engaged in DNA synthesis. The reutilization pathway is of considerable magnitude. We do not know yet whether thymidine is present as such or whether it is in the phosphorylated form in the reutilization pathway. Hughes estimated that the effective thymidine pool in the total mouse was 0.05 μ mole; it turns over rather rapidly.

SOME PROBLEMS ON THE BIOLOGICAL
USES OF TRITIATED THYMIDINE

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Although this colloquium is concerned with certain problems in connection with the use of tritiated thymidine, I want to emphasize that they are not necessarily problems connected with tritiated thymidine but thymidine in general. One of the remarks mentioned in the discussion following the last paper was the question of chemogram and I think that this is a sufficiently non-specific problem to be mentioned first of all, and to say that chemogram indeed does exist, and that it depends on tissue and experimental conditions. We have seen occasional strains of Ehrlich ascites cells, for example, which gave a beautiful "autoradiograph" although it came from preparations and animals which did not receive any radioactivity. On repeated experiments this was proved to be a chemogram occurring in certain strains of mice only. The chemogram effect was on one occasion so strong that we did not have to develop our films, it was enough to put them straight into fixer after a few weeks of exposure. This reducing activity was removable by washing the cells for 15 to 29 minutes with trichloroacetic acid.

To come to radioactive thymidine the first question is that of the radiation effect, but I am not going into this detail because this will be discussed by other speakers later. However, I want to call your attention to the fact that tritium, in the form of thymidine, is a unique form of irradiator for the cells. Intracellular radiation, or perhaps more correctly, intercellular radiation does occur with any label in any compound which we administer to cells, but even including ^{14}C, most of the radiation received by a cell will be a radiation from a particle emitted by *another* cell. With tritium we are in the unique situation that the radiation received by the cell will be emitted by that very same cell because tritium has an average range of about 1 μ. It should be remembered, however, that in this volume of 1 μ diameter sphere a total dose equivalent to about 170 rad can be dissipated. Whether this sphere happens to be an important or unimportant site in the cell, only the biological effect will say.

The second effect of radiation is a question of transmutation — what a DNA molecule does when it finds itself suddenly with helium instead of hydrogen. What is remarkable is that considering the amount of radiation received by the cells and the amount of transmutation, the biological damage is extremely small.

The second point which was also mentioned in the earlier discussion is the problem of re-utilization. The whole question of re-utilization of nucleic acid precursors, especially for DNA, was discussed by Hamilton many years ago, but he was primarily working with purine precursors. Purines are undoubtedly re-utilized,

and very efficiently too. We know that we can keep people on a completely purine-free diet. We know of the excretion of purine compounds in urine, and whether a person is on a normal purine diet or a completely purine-free diet, the total excretion figures do not drop by more than a factor of two. If you calculate the amount of new purines which have to be synthesized to provide all the growing cells in the body, there is a great difference and defect compared with the amount newly synthesized by the liver, the amount which can be synthesized by the cells, especially in view of the fact that bone marrow cells, for example, cannot synthesize purines, and it becomes clear that re-utilization is very efficient indeed. The question is, is there a similar efficiency of re-utilization of thymidine? We have labelled mice — both with ^{14}C adenine and tritiated thymidine — and then determined the DNA specific activity of the bone marrow cells at various times after injection. As the DNA is being synthesized days and days after administration of the original label, the specific activities should be diluted. These are the so-called dilution curves on which population growth studies are sometimes based.

In our experiments, the two slopes — the slope for the DNA specific activity for tritium, or the DNA activity for ^{14}C — differed insofar that they were steeper with tritium than with ^{14}C. This means that while there is a re-utilization of purines, the re-utilization of the thymidine is definitely much less efficient. The trouble is that we cannot very easily get a pure baseline of a situation where there is no re-utilization whatsoever.

The third problem is the question of DNA turnover. This is a question which in the ears of many is equivalent to blasphemy. DNA is supposed to be an inimitable, non-turning-over substance which is laid down once and for all in a semi-conservative fashion with the result that some of us, with a bit of luck, may have in our bodies DNA molecules first synthesized 50,000 or more years ago. However, I think that Dr. Pelc certainly would agree with me that there is now a considerable body of evidence indicating that in certain cell types, there is a limited turnover or exchange of DNA even when the cell is not in a state of cell cycle. What exactly this turnover means — whether it is an intracellular repair of certain mechanisms going wrong during DNA duplication, or whether it is a certain mobility of single-stranded parts of the DNA double helix — remains to be seen, but it is certainly a problem which one has to consider, especially when one is dealing with autoradiographs and grain counting.

Then come a whole host of various metabolic headaches. One metabolic headache, of course, is the pool dilution problem and pool effects. We first noticed this some years ago when we irradiated Ehrlich ascites cells with various doses of radiation. Some will remember that there was a time when the magic figure of 50 % depression was found by a large number of systems in the range of 500 to 5,000 rad of radiation *in vitro*. For quite a while we also got very repeatable 50 % depressions with 2,000 rad of the thymidine uptake, but one black-looking week, the depression dropped down to 10-12 %, and for weeks and weeks afterwards it was 10-12 %, and we could not get a 50 % depression with 2,000 rad. When we looked carefully

at the experimental details, we found that in these experiments the amount of thy-
midine administered to the mice was greater by a factor of 100 (compared with our
earlier work) simply because a lower specific activity sample of thymidine was used.
Clearly, what must occur is that with the high specific activity compound, we are
dealing with a pool dilution in that some of the cells irradiated released enough
thymidine to be comparable in amounts to our label, while with the low specific
activity compound our label overfloods the endogenous pools.

Another problem is the decomposition of thymidine in the medium. We noticed
that in some of the bone marrow cultures there was a curious discrepancy between
the grain counts and their increase in time, and the amount of thymidine added.
By varying the specific activity of the compound one would expect to be able to
predict the grain counts and their increase in time. These predictions, however, never
worked out in practice, and the only possible explanation was that some of the
thymidine, which was added to the medium was decomposed in the culture medium.
This was very irregular in these bone marrow cultures, but later Dr. Cooper in
London, repeated similar work with phytohaemagglutinin-treated blood cell cultures
and found the same, i.e. that thymidine is decomposed in the medium. He also
found that the degree of decomposition is more or less related to the amount of
granulocytes in the culture medium.

The next problem is the question of feedback control of thymidine uptake
and the question of transport versus incorporation and non-specific absorption.
The Chester-Beatty group of workers have shown that the amount of TTP had
certain feedback control effects on the kinase activity and may, therefore, block
further incorporation of thymidine into DNA. This means, of course, that we will
have the thymidine itself, being an extremely soluble and diffusible substance, in
equilibrium between the medium and the cell, but not getting into the high polymer
states. The question can be asked, however, what form is the label in the cell which
we see on autoradiographs ? Crathorn and Shooter have found a curious discrepancy
between the amount of DNA label as judged from autoradiographs, and DNA
specific activity as judged from biochemical isolation of DNA; and in each case the
autoradiographs indicated a higher DNA labelling. Of course, DNA by autoradio-
graphic definitions is only removable by DNAse and not by the mild acid hydrolysis.
On the other hand all this is not high polymer DNA, if high polymer DNA is defined
as that which can be removed by the usual biochemical isolation techniques. If we
are defining DNA as that which is biochemically isolated from the cells, then the
autoradiographic data mean that there is a non-specific absorption of label in a
form which is not removable from the cells by any kind of fixing. One of the effects
of irradiation of the ascites cells was not so much the depresston of the absolute
rate of DNA synthesis in the cell, but the rate of transport of the thymidine into the
cell.

Finally, to take the evidence of chromosome labelling studies we know now
that during chromosome duplication there are certain parts of certain chromosomes
which are very "hot" at certain times during the S-period of the cell, but "cold"

before or after this time. "This time" is a relatively short period of the total seven hours of the DNA duplication period (S-period) of the cell as a whole. What the chromosome labelling patterns show is that we cannot speak of S-period for the cell, except by knowing that this is a vague generalization, and that we ought to be talking about S-periods of parts of chromosomes. We must remember that DNA is synthesized in discreet chromosomes of which there are 46 in human cells, and even within these chromosomes DNA is synthesized in a discontinuous fashion in any particular part of the chromosome, and there appears to be a strict time sequence of which the chromosome autoradiographs are still a crude generalized representation. The labelled bands are visible only because at that time, by chance, on that chromosome, a larger number of genes happen to be duplicating in close proximity to each other. Although any one gene duplication may take only a minute or seconds, the whole sequence is spread in the cell over 6-8 hours. This means, of course, that to look upon DNA synthesis and DNA duplication in the cell as a purely biochemical process is a gross misconception, because what must happen, of course, is not only for precursors to be there, but for the DNA to split at the right time and place. This sequence may have certain evolutionary significance, of course, as I should imagine that while a particular gene is duplicating it cannot code and there may have been a very important survival value in determining the sequence when a cell can afford one particular locus, not coding, in relation to other loci which might be duplicated.

The problems cited above are only a sample of the kinds of difficulties we encounter in interpreting labelling experiments with thymidine, but of course not all of them are specific for thymidine. As research progresses we manage to solve many of these problems but not without creating some new ones.

DISCUSSION

PELC : I quite agree with you that this discrepancy between biochemical findings and autoradiography is very disturbing.

Shooter has found that by autoradiography more can be scored than by biochemistry, but we now find some very serious discrepancy the other way where the biochemist finds much more labelling than we do. The most clear example is striped muscle. Drs. Gerber and Altman found 40 days turnover time for striped muscle, we find about 200 days. In other words, we seem to miss something there.

This simply means that different parts of the DNA are scored by the two techniques. One possibility I could think of is cytoplasmic DNA and very low labelling DNA. There is DNA in mitochondria and biochemist will score this as labelled DNA as well. Not enough is known about labelling of mitochondria to say anything definite about it. But it is a very disturbing thing altogether.

GERBER : In the case of muscle, we compared autoradiographic data with data obtained from measurement of specific activity of DNA. I have the impression that certain cells, such as monocytes, endothelial cells and some leucocytes carry most of the label. Similar experiments were done with dystrophic muscle where other people found a considerable increase of DNA synthesis and DNA turnover. We found in muscle from muscular distrophy also a great number of such cells due possibly to the existence of inflammation areas in the muscle.

LAJTHA : This is why I feel that autoradiography is very essential counterpart and check in a biochemical work. I try to remember who published this very interesting experiment where they prelabelled an animal. The labelling of course will not produce any significant label in a normal resting liver. But after steady state has been achieved, several days after labelling, partial hepatectomy is done, and suddenly quite a large number of labelled cells will appear in the liver. If you do only a biochemical estimation of DNA specific activity in this system, you get a terrible surprise. Where does the thymidine come from ? There must be a terrible re-utilization.

When you look at the autoradiography you will find that some hepatocytes are labelled, very few, but there will be a significant infiltration of small round cells, which of course belong to this lymphatic pool which has been labelled earlier and which might migrate into the damaged tissue. That of course is not re-utilization, it is redistribution of labelled cell population, immigration of labelled cells because you create pathological condition. But these small lymphocytes have been accused of disintegrating themselves and giving large chunks of their DNA to other needy cells. Some of the hepatocytes labelling may come from that source. It is very important for both techniques together.

PELC : Muscle nuclei were checked very carefully and so we scored as to the best of our knowledge only muscle nuclei. We leave out labelled endothelial cells

and lymphocytes and so on. So, I don't think that this question can be solved on the idea that only some cells which happen to infiltrate are responsible for it.

GERBER : I have a question to Dr. Lajtha. You suggested that perhaps what we determine in terms of grain counting is not the same as what we isolate by chemical extraction as DNA (Schmidt-Thannhauser or Schneider technique). I do not quite see how this would be possible since in many cases you actually follow very similar procedures. You treat the nuclei with ethanol and later with perchloric acid, and then you measure all that is in the rest. This can only be DNA, RNA or some proteins. But it is known that not much remains in the residue if you extract with hot trichloroacetic acid.

LAJTHA : It is a fact, and if you isolate DNA biochemically and you do very carefully total cells counts and you know how many cells you had and how many counts you had for this cell population, and you do the same thing with autoradiographic procedures or the same by chemical procedures which would be applied to the cells which you autoradiograph, there will be a discrepancy. This discrepancy may be varying in degree : there is very little discrepancy in bone marrow cells, quite marked in the Yoshida cells and in some of the Ehrlich cells. The degree of the discrepancy may be practically undetectable and it becomes obsessional to look for it as meaningless. But in some cell systems it may be really quite remarkable and there, something is wrong and I suspect that what is wrong is our definition of what is DNA.

BRESCIANI : I would like to make a remark on the last point of Dr. Lajtha, about the rate of DNA synthesis during the S-period, according to some of our data from a mammary gland experiments.

We plotted the average number of grains after pulse labelling with thymidine, versus mitoses, at different times after the injection. Dr. Lajtha said that this is a method for measuring the synthesis of DNA at different times during the S-phase.

According to our data there is not a constant uptake of thymidine during the S-period : there is first a steep increase, then some kind of plateau and then slowly the uptake of thymidine into DNA decreases.

Plotting these data in a tridimensional way, one can see in these cells that at first there is a slow uptake of thymidine into DNA, then there is a second phase of quite a large uptake and then slowly the uptake decreases and there is a final phase of low uptake. There is also overlapping of these different phases. We have calculated what should be theoretically the expected grain count distribution on nuclei in the cell population and confronted with what we actually get in the mammary gland.

Generally, we have a kind of distribution which cannot be interpreted as a Poisson distribution.

As a matter of fact this distribution has a shoulder on the left and there is also a number of cells which have a very low grain count. This cannot be interpreted as a Poisson.

According to our results and according to these analyses, it is a mixture of Poissons, every Poisson's having the average as shown in the first curve I showed you, that is, the rate of DNA synthesis during this phase.

LAJTHA : As far as the Poisson distribution is concerned, I remember some years ago we studied the grain counts distribution on the basis that this should be a measure or a potential measure of the rates of uptakes per cell in a cell population, and if the cell population is homogeneous, and the rate of synthesis is the same in all the cells, then the grain counts should be a Poisson distribution. This we only found in nondividing cells. In small lymphocytes given some protein precursor or some nucleic acid precursor, we can find a very nice Poisson distribution predictable from the grain counts. By any growing cell population, whatever label we gave, protein, RNA, ^{5} Fe, it was not a Poisson distribution, but of course you have to realize that those cells are growing cells which means that they have to increase the cell mass from the postmitotic true diploid state to the premitotic tetraploid state and the cell mass will increase all through that time. What the cells guard very jealously more than anything else is the concentration of dry material per unit volume of cell and the concentration of dry material of course remains exactly the same all throughout cell cycle, meaning that before mitosis the cell has twice of everything than immediately after mitosis.

The question is how is this increase in mass occurring ? Is this a linear increase per unit time or is it an exponential ? As the range is two to one, to differenciate between linear rate of increase and exponential is very difficult and is highly theoretical, but when you plot a theoretical grain count distribution on this increasing rate exponential or linearly then you get a distribution curve which is exactly the same as that you find in the experimental data.

The agreement was so good that we were unhappy about it for months, because when predicted and found experimental curves fit, there is something suspicious. But this was not the case with DNA. With DNA the distribution was very much wider and the range was not one to two, but one to four, one to six and even that was an approximation and the only explanation is what you just said, that it is an overlapping Poisson for that segment all the time. This is specific for DNA.

FEINENDEGEN : Answering the problem of specificity of autoradiography in comparison to biochemical data. If one compares the results of the two and does not find agreement, is it not also possible that the type of fixation for autoradiography of the tissues might extract the DNA ?

I think Bateman, Post and Hoffman's studies on toxicity of tritium on the ploïdy of liver cells showed that ploïdy change is effective also there where there was no label in autoradiograms, so they assumed that the labelling was lost during the preparation of the section for autoradiography. The second point in explaining these discrepancies may perhaps be coincidence errors. If one looks on molecular dimensions and on a sequence of thymidine molecules in a DNA chain and looks at the diameter of a grain, it is just like an umbrella into which are shooted small

pieces of dirt; one grain may be representing more than one label family because a second hit in the emulsion would not be registered as a radioactivity. So in some cells types the coincidence error might help to explain the discrepancies one definitely observes.

LAJTHA : For this reason it is vitally important to know the grain yield per incident electron in any autoradiographic experiment.

PELC : For stripping films grain yield is 0.85 grain per β particle hitting the film. This is a very constant figure actually, it does not vary by more than 10 %. The density can vary considerably more but the grain yield does not.

FEINENDEGEN : I am thinking not in terms of the sensitivity of the silver halide crystal but in terms of the geometry of the grain size and sequences of thymidine molecules in the DNA chain.

PELC : Do you mean local saturation of the film ?

FEINENDEGEN : Yes.

PELC : We got the same discrepancy by circumventing grain counting, because we said that if the grain count say in a crypt cell and in a cell in another organ is approximately the same, we can assume that the rate of synthesis or the rate of incorporation will be the same. Therefore by counting the number of such cells which presumably double their DNA content, we should still get a valid comparison with the biochemical results. These are discrepancies up by a factor of ten. I think the explanation must lie deeper than in just technical reasons.

SCHULTZE : In our laboratory, Dr. Koburg found Poisson distribution of the grain counts over interphase nuclei in certain tissues. From this we assume that the DNA synthesis rate should be the same for different cells and that if the DNA content is the same, then the synthetic phase must also be the same (it has been shown to last 7 hr for many tissues). He also found very good curves of grain counts over the mitoses : a steep rise, then a plateau and then a not very steep fall. I think this has been confirmed by Pilgrim with a double labelling technique : he injected first tritiated thymidine and 2 hr later ^{14}C-thymidine and vice versa, so he could locate the cells which are in the beginning of the S-phase and in the end. And during the four hours, the two hours of the beginning and the two hours in the end, he always found the same grain count in the interphase nuclei. In the mouse at least it seems that there is quite a constant rate during the S-phase.

LAJTHA : I remember these curves of Dr. Koburg. When he first came out with them there was a violent argument between him and the late Henry Quastler who found a very much wider distribution and certainly not Poisson.

Now Lamerton has a very good system by which he can tease out cells from the crypt so that they are very nicely spread and he can do grain counts on them, and

they are also not Poisson. Why in a concentrated fixed tissue the grain counts should appear Poisson and in others not, I don't understand. It is not a general phenomenon.

SCHULTZE : If you have a squashed preparation, I think that the geometrical factors are not as good as in sections.

LAJTHA : I do not think that geometrical factors come in, because the same thing has been repeated with ^{14}C-thymidine, where geometry is not a problem at all, and again it does not give a Poisson.

SMITH : I would like to change the subject from that of cellular level into that of the intact organism. In your talk you did show 2 curves, one of them involved intravenous injection of tritiated thymidine and the other peritoneal injection. I wonder first of all, what activity you injected into these animals and also the mass of cold thymidine which you put in ? The other point I would like to know is, what would happen if you flooded the system metabolically with cold thymidine. And finally would you like to say the turnover of thymidine in a rat per day ?

LAJTHA : For the latter I don't know. For the first we gave in both cases half of a microcurie per gram bodyweight of the high specific activity compound approching two millicuries/μ mole.

As far as the turnover is concerned, there will be of course a very marked difference in turnover between various tissues due to simple cell population turnover. Also various tissues will have local concentrations of nucleases, DNAse, especially after some injury, and I am thinking particularly of Peter Alexander's results in the thymus and liberation of nucleases there compared with the spleen. After identical whole-body irradiation, we have a vast increase of nucleases in thymus but very little in spleen.

A localized head radiation which scatters over the thymus in a small rodent is enough to liberate a large amount of these nucleases which of course will result in DNA breakdown in a number of non-labelled cells.

PELC : As to the question of total turnover in animals, we find that the very fast dividing organs like intestinal tract and bone marrow have a turnover time of the order of one and a half to two days; skin has a turnover time of the order of 20 days. The non-dividing organs have a half-life between 20 to 50 days.

We have to bear in mind that non-dividing organs are much bigger than the dividing organs. I did a calculation and approximately equal amounts of thymidine are incorporated into muscle and liver, as compared with the dividing organs, simply because of the large difference in size. So the average turnover time in the whole animal will come to ten days or so. That's a very rough figure and not accurate.

GERBER : One of the big difficulties of course, is that we don't know how much thymidine is synthetized. We can only estimate how much has been metabolized

from DNA, but the amount of synthesis and how much is directly catabolized, we cannot estimate. We have some data on liver which we shall show tomorrow, but from these data it seems almost impossible to flood DNA thymidine catabolism, because even if you add as much as 5 milligrammes of thymidine to a perfused liver, the liver catabolized it still almost at the same rate as its normal rate for 5 γ of thymidine.

This brings up another problem which belongs more to the topics we discussed before. We have at the same time also studied incorporation into DNA by chemical methods and it seems that there we find also a difference versus autoradiographic means. Then we studied incorporation of thymidine DNA as a function of the amount of thymidine given : we find a very slight increase in the number of grain counts as the late Quastler has shown in a very nice model. Now it seems that we found a much larger increase in incorporation into DNA isolated by chemical methods. I have not as yet an explanation for this and we have to wait for more experiments.

ALTMAN : I would like to ask a question concerning the uptake of thymidine by mammalian cells. You made a point of saying that very often when you studied radiation effects, you study in part an effect in the uptake by the cell. Now I wonder if you have any thoughts about the mechanism and the controlling factors of the uptake because this must be of very great importance in determining whether or not the cell is going to take thymidine or not, for example, is it possible that in a course of transit across the envelop of the cell, phosphorylation takes place as we know it does occur in the case of sugars ? Is this a controlling factor in the uptake of thymidine in your opinion ?

LAJTHA : First of all I would reiterate again the question of pool dilution both local and general. We can, with irradiation, release nucleases and this of course will greatly affect the uptake without affecting the synthesis. Secondly, what these phosphorylating enzymes are and how they are affected by radiation which comes into this pathway of phosphorylating thymidine (which is a side pathway coming into the main natural pathway), I don't know. But I know that there are some cells, especially these small lymphoid types of cells which are fantastically radiosensitive in respect of one phosphorylating enzyme (TMP to TTP) and this enzyme activity can be destroyed by as little as 50 to 75 rad of radiation as shown by Stocken. If these enzymes have an effect on that side pathway pushing thymidine into the main deoxyuridylic acid pathway they will greatly affect uptake without affecting DNA synthesis.

ALTMAN : Some of these enzymes are located in the nuclei of the cells. Is there any evidence for kinases (the phosphorylating enzymes) to be localized near the surface of the cells ?

LAJTHA : I don't know, but there is another effect which comes in and that is the so called G1 delay, and this has nothing to do with the rate of DNA synthesis.

If we give 400 to 500 r to a regenerating liver, at a time when DNA specific activity is already rising, nothing will happen to those cells which are already in a state of DNA synthesis, they will synthetize at perfectly normal rate whichever way you measure the specific activity. But those cells which at that time are still in the G1 period will be feeding into S at a very decreased rate, and the kinase activity will be depressed very significantly.

HOLLDORF : You mentioned the feedback inhibition by TTP of conversion of thymidine to TMP. There are at least 2 other known processes in the whole sequence where TTP acts as a feedback inhibitor. First, the deaminating step from deoxy-cytidine monophosphate to deoxyuridine monophosphate, and secondly the reducing step from ribosyl to deoxyribosyl-compounds. We should consider which of the three processes is feedback inhibited. There is a feedback inhibition on the precursor reutilization from thymidine and there are at least 2 other feedbacks in the *de novo* synthesis. Which is the right one ?

LAJTHA : Partly the answer could come from comparing thymidine labelling and its sensitivity with labelling by deoxycytidine or deoxycytidine monophosphate. Dr. Cooper has shown that while thymidine is decomposed in the medium, deoxy-cytidine monophosphate is not, and deoxycytidine monophosphate is certainly on the main pathway.

I would like to see at some stage some good comparative study between these two labels.

HOLLDORF : For *E. coli* we have such data, and I will present them tomorrow but the point is that in *E. coli* there is no pathway from deoxycytidine monophosphate to deoxyuridine monophosphate. There is a completely different pathway here, and this pathway is feedback inhibited.

FIRKET : This is more or less a related question to the preceding ones. Has Dr. Lajtha or anyone data on the efficiency of the use of the added thymidine in producing the thymine included into the DNA ? What percentage of the total thy-mine in the polymerized DNA can be made up from the thymidine added ? I remember several years ago I did some calculations on this in tissue culture and my figures brought me to about 50 to 70 % counting on the efficiency of grain develop-ments and s.o. but I never believed very much in my calculations. Has anyone serious data on this ?

LAJTHA : I have made similar calculations and after a while* I got rather dis-paired, because these calculations varied from system to system, specific activity, amount and time, time in particular. I don't think I ever got up to 70 % replacement of thymidine, I think my highest calculated figures were about the order of 20 to 25 %. But there is a great error in these data. We have to consider specially in *in vivo* experiments local differences in the rate of blood flow through various organs.

Because of the extremely short plasma clearance of thymidine after intravenous injection, this will create great local differences in concentrations at any time, and that in fact will produce great differences in grain counts.

In vitro it will depend on cell types even in one so called suspension like bone marrow cells : the efficiency will be greater for the normoblasts than for the myelocytes, even, assuming similar S-periods for the two. Any possible difference in the S-period is smaller than the grains taken up by them. Again it will depend on the number of granulocytes in the bone marrow suspension. The differences do exist and I think these differences are primarily in *in vitro* conditions, cell type and condition of the cell.

FIRKET : May be it is due to the amount of the pool of the natural precursor.

LAJTHA : I don't think so because you can, under good conditions, increase the pool *in vitro* into vast amounts.

GERBER : When I was last year in Madison I was shown some data by Van Lancker which showed that by certain doses of radiation, you can inhibit DNA synthesis but don't have an effect on thymidine kinase. As far as I know there was a recent paper by Bazerga where it was shown that the uptake of thymidine into DNA was of the order of 8 % for the total animal. We find in a perfused liver for normal liver approximately 0.1 to 0.5 % of the total dose; for regenerating liver between 5 and 13 %, and in case of perfused intestine within one hour we find in the order of 8 to 10 % of the thymidine added.

LANG : We have injected several mice with doses of 50 microcuries per mouse and we have isolated the DNA by the Schmidt-Thannhauser method and measured its specific activity, and we found that it had only 1 to 3 % of the specific activity it could have if only the thymidine of the injected material was incorporated in the DNA. And in cell cultures studies the percent is not much higher than in the animals. I can also give some data to the remarks of Dr. Gerber. We have found in the small intestine 10 to 15 % of the injected dose of thymidine. And in all other organs which we have examined, lungs, submaxillary gland, and pancreas, below 1 % of the injected dose was incorporated.

GERBER : The data I was referring to were in a perfused intestine, where we found in the order of 8 to 10 %; the data of Bazerga and of the total animal I was referring to were of the order of 4 %; that is about 50 % of the total amount of thymidine incorporated into DNA is found in the intestinal tract.

EXEMPLES DE RÉSULTATS CONTRADICTOIRES OBTENUS EN EMPLOYANT DE LA THYMIDINE TRITIÉE EN AUTORADIOGRAPHIE

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Il n'est pas question de développer ici l'ensemble des difficultés que soulève l'emploi de la thymidine tritiée. Selon l'objet prévu de ce colloque, je présenterai deux exemples seulement, issus de mon laboratoire et qui pourraient situer les principales sortes de contradictions auxquelles on peut se heurter dans l'emploi de la thymidine tritiée. Bien d'autres laboratoires sont d'ailleurs en mesure de fournir des observations analogues.

Le premier exemple est relatif à l'étude de l'ovogenèse chez la drosophile. Dans ce cas, deux séries de résultats entièrement différents ont été observés les uns en 1959 et 1960, les autres à partir de la fin 1960. Ces résultats sont résumés dans le tableau suivant :

	1959-1960	1960 et après
Marquage sensible à la DNAse :		
à situation nucléaire	+	+
à situation cytoplasmique	+	—
Marquage sensible à la RNAse :		
nucléaire	+	—
cytoplasmique	+	—
Marquage insensible aux enzymes	Faible	Très faible

Les premiers résultats avaient été obtenus de façon très régulière. La seconde catégorie de résultats a été obtenue de façon aussi constante; malgré des essais comportant les modalités les plus diverses la première sorte de résultats n'a pu être obtenue à nouveau [1, 2].

La seule différence qui semble exister entre les deux catégories de résultats tient à la différence des lots de thymidine employée. L'une des hypothèses consisterait à admettre, pour expliquer ces résultats, que la première catégorie de thymidine ait été souillée par un dérivé nucléosidique tritié susceptible de s'incorporer dans l'ARN. Or, il se trouve que ces mêmes lots de thymidine ont été employés pour étudier le métabolisme des acides nucléiques dans la glande séricigène du ver à soie. Ils n'ont fourni alors qu'une incorporation exclusivement nucléaire malgré l'importante synthèse d'ARN qui se déroule dans ces glandes : la présence d'un nucléoside

tritié autre que la thymidine semble donc pouvoir être exclue. L'explication la plus vraisemblable paraît être celle d'un marquage sur la fraction glucidique de la molécule de thymidine. Celle-ci étant en partie clivée dans l'organisme a pu être incorporée dans les constituants les plus divers. La différence entre les observations sur la drosophile et celles du ver à soie pourrait résulter du fait que, compte tenu de la taille très différente des animaux, le ver à soie a reçu une concentration de thymidine tritiée beaucoup plus faible, soumise donc à une dégradation quantitativement moins élevée.

Le second exemple est relatif à des nématodes libres du genre *Cænorhabditis*. Ceux-ci reçoivent la thymidine par l'intermédiaire du milieu dans lequel ils sont élevés. Ce milieu est rigoureusement axénique afin d'éviter la dégradation de la thymidine par des microorganismes. Il contient, outre des acides aminés, vitamines, peptones, un extrait brut de foie dont la composition est mal définie. Dans un premier ensemble d'essais employant une certaine préparation de foie, on a obtenu un certain tableau de marquage. Celui-ci comportait, en particulier, après 24 heures d'incubation, un marquage nucléaire dans les noyaux de l'intestin, noyaux de grande taille, vraisemblablement polyploïdes, qui, chez cet animal, ne subissent pas de divisions. Dans une seconde série d'essais employant une autre préparation d'extrait de foie, on a retrouvé les principales caractéristiques du marquage antérieurement observé, à l'exception du marquage des noyaux de l'intestin [3, 4]. Un examen de l'évolution de la thymidine dans le milieu montre que, dans le second cas, deux heures après l'addition de la thymidine, celle-ci est, pour les 9/10, réduite à l'état de thymine. On s'explique ainsi qu'aucun marquage ne soit observé dans des noyaux dont le métabolisme nucléique pourrait être excessivement lent. Ce second cas constitue donc un exemple de variation due à des causes d'ordre biologique. Ces exemples pourraient être multipliés et la communication du Dr Lajtha en a dressé ce matin un tableau complet. Leur étude fait l'objet des investigations du biologiste.

Les contradictions relevant de la nature des préparations de thymidine employée, au contraire, devraient normalement échapper à notre analyse. Lorsqu'elles se produisent, elles contraignent les biologistes à des travaux qui ne sont pas nécessairement de leur ressort. En conséquence, il devrait être possible de demander aux fournisseurs de molécules marquées une fiche d'identification de leurs produits plus précise que celle qui est actuellement fournie. Des efforts importants ont déjà été accomplis dans cette voie par les principaux fournisseurs, mais ils restent insuffisants. Une liste non limitative des indications souhaitées pourrait être la suivante :

1) Date de l'analyse effectuée et conditions de conservation employées depuis cette analyse ;

2) Définition du degré de pureté chimique (avec indication de la méthode employée) ;

3) Répartition en pourcentage du tritium sur le sucre, sur le cycle, sur le 5-méthyl (avec indication des méthodes de mesure employées).

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DISCUSSION

VAN DEN BROEK : We got quite similar results with invertebrates. Working with *Asellus aquaticus*, we observed incorporation of ^{3}H-thymidine in the nuclei of the intestine after 3 hours; now this intestine does not divide.

We were also interested in the development of the oocytes. After ^{3}H-thymidine injection to the females, we found that the yolk granules, quite abundant in the eggs, are heavily labelled in the centre. The position of the label varies according to the time between injection and observation.

^{3}H-thymidine is therefore incorporated in the yolk granules in successive layers as the oocyte grows. This incorporation is DNase-resistant and RNase-resistant. We tried chemical extraction on 1,500 eggs put together in a tube. After fat extraction and trypsinization of the eggs, one gets an extract having a high OD at 260 mμ. DNase treatment followed by chromatography does not give anything. Alkaline hydrolysis in 0.2 N KOH gave one unidentifiable spot. Perchloric acid hydrolysis gave only some adenine and guanine. In the injected animals, however, all other somatic cell nuclei are labelled and behave normally after chemical treatments.

If we let the eggs develop after injection, we observe that the label of the yolk granules is used and concentrated in the young embryo cells.

I can offer no explanation as to the nature of the labelled compounds in this case.

NIGON : Cela me rappelle les observations de Durand.

VAN DEN BROEK : Yes, but Durand found label also between the granules. We find it all through the granules since we section them. Every granule is labelled homogeneously; not all of them are labelled to the same extent, but they are all labelled homogeneously.

NIGON : C'est certainement un remaniement de la thymidine et il n'est pas nécessairement en rapport avec une synthèse d'acide nucléique.

VAN DEN BROEK : On perd toute la thymidine tritiée par un traitement normal histologique.

NIGON : Et si elle est fixée dans des dérivés protéiques ?

VAN DEN BROEK : Ils seraient fixés très fortement. Après fixation par l'alcool acétique, ils seraient perdus, je pense.

NIGON : Dans la mesure où la thymidine tritiée peut être remaniée et entrer, par exemple, dans la composition de l'alanine, il me semble qu'il y a des possibilités de voir entrer le tritium dans des acides aminés; après il est fortement fixé.

VAN DEN BROEK : Mais cela n'explique pas qu'après 14 jours on trouve toujours l'incorporation dans un composé absorbant à 260 mμ. J'ai pensé que, peut-être, il s'agit d'une autre sorte de polynucléotide très dense, comme dans le sperme.

PELC : At what time do the embryos take up thymidine again ? They utilize the thymidine in the yolk to synthesize DNA for the first number of divisions. If at the 8 or 16 cells stage tritiated thymidine is offered, do they utilize it as well or not ?

VAN DEN BROEK : They do utilize. As far as I know they do.

NIGON : We can give tritiated thymidine to eggs by injecting the females 2 hours before oviposition. And the labelling goes in the cytoplasm but as tritiated thymidine. And then the embryos nucleide fix this thymidine.

PELC : Have you considered the question of mixed nucleic acids consisting of RNA and of DNA ? Such hybrids have recently been synthetized in the laboratory and may be these compounds are such mixed compounds that might explain some of your findings. We do not know anything at present about chemical properties of such mixed nucleic acids.

VAN DEN BROEK : We have tried to hydrolyze them and see if there is some thymidine. But we did not find it. It is the only answer I can give. Biochemists have tried to see — we have thought about the combination RNA-DNA. You would expect to have a really strong hydrolysis either in the alkaline side or the acid side. You would get some of these compounds back. But you don't get them.

KROGER : Did you use instead of thymidine sometimes cytidine and looked if you have the same effect with cytidine. On the other hand, you should perhaps heat your isolated material for if you have a hybrid you should dissociate this hybrid by heating and then it should be split by DNase or RNase.

VAN DEN BROEK : We did not try heating them. The first slide I showed was really from eggs incorporating cytidine and not thymidine. But we get exactly the same results.

SMITH : In view of the chemical resistance of your tritiated thymidine what evidence have you that it was in fact incorporated into DNA.

VAN DEN BROEK : The only thing that we know is that the normal somatic cells behave normally. This is the only thing we know, we only see that there is incorporation and we don't know what form. I don't say it is incorporated into DNA in the Yolk granules. We find incorporation, and we wonder what it is.

PELC : In crabs, a very curious DNA has been found which consists of adenine and thymine only. Anything known about the chemical properties ?

One more question about the incorporation in the intestinal tract, the incorporation you find in non-dividing cells. We have plenty of evidence now for renewal of DNA in non-dividing cells and it seems that they are some organs which maintain themselves by cell division and organs which cannot divide for one reason or another, but simply renew their DNA.

Do you think this might be one of these cases ?

NIGON : I must say I would be interested to find if this could be a clear cut case of DNA renewal.

PROBLEMS OF THYMIDINE REUTILIZATION
AND TOXICITY OF TRITIUM RADIATION
IN THE BONE-MARROW OF RATS (*)

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Renewal of deoxyribonucleic acid (DNA) in a cell population is an important parameter for studying various influences on the mechanism of DNA synthesis and on cell proliferation. Isotopically labelled thymidine, particularly ³H-thymidine, has been extensively used in such investigations.

In this communication some data on the validity of ³H-thymidine as specific precursor for studying DNA renewal is discussed with emphasis on the problems of reutilization of DNA-thymidine and of the level of toxicity of ³H-thymidine. In principal, both of these have been recognized over the past few years [1]. But it has not been attempted yet to define in a given tissue the magnitude of the physiological reutilization pathway and to distinguish it from effects possibly induced by the radiation from tritium incorporated into DNA with thymidine. A clarification of these situations is of interest not only with regard to the interpretation of chemical and autoradiographic data on cell proliferation and DNA renewal, but it bears also on the question of possible implications on the functions and genetic expression of single cells within an organ [2].

MATERIALS AND METHODS

The experiments presented here were carried out on the bone marrow of Sprague-Dawley rats, 3-4 months old and about 200 g in body weight. The following precursors for DNA were chosen : ³H-thymidine (³H-Tdr), specific activity 1.9 C/mM (from Schwarz Bioresearch, Mt. Vernon, N. Y.), 2-¹⁴C-thymidine (¹⁴C-Tdr), specific activity 30 mC/mM (from New England Nuclear Corporation, Boston), and 5-¹³¹I-2'-deoxyuridine (¹³¹I-DU), which was kindly prepared by Dr. Commerford, Brookhaven National Laboratory, according to the method of Prusoff [3] to a high specific activity above 1 C/mM. These precursors were injected intravenously in the following doses per gram body weight : ³H-Tdr 0.1, 0.5, 1 μC; ¹⁴C-Tdr 0.01 μC; ¹³¹I-DU 0.18 μC.

As indicated under results, the animals were killed at various times after injection, and the femurs and tibiae were cleaned from soft tissue for obtaining the bone marrow for chemical and autoradiographic evaluation of label incorporation.

For the chemical measurements, the cells were dispersed in saline, counted in triplicate samples, and transferred in a precise volume for further work-up. The acid insoluble fraction of the cells was taken to represent DNA label only, after verification of the specificity of the precursor incorporation into DNA [4]. The ^{3}H and ^{14}C was measured by liquid scintillation counting, and the ^{131}I was assayed in a crystal well counter.

Autoradiograms were prepared from bone marrow smears on microscopic slides after fixation in methanol, with NTB$_2$ Kodak emulsion [5]. The finished auto-radiograms were stained with Giemsa (pH 6.0) and evaluated microscopically for the number of grains per cell, differential cell counts, and for mitotic indices. A complete set of cytological data was obtained from the animals injected with 1 μC ^{3}H-Tdr per gram body weight.

<div align="center">RESULTS AND DISCUSSION</div>

1. THE ACID SOLUBLE POOL OF THYMIDINE

In order to assess properly the data on thymidine reutilization, some remarks on the acid soluble pool of thymidine or its derivatives shall be made first.

^{3}H-Tdr has been shown to become incorporated into the acid soluble fraction of only those bone marrow cells which are known to be able to synthesize DNA [6]. Thus most mature cells, such as segmented neutrophilic myeloid cells, orthochromatic normoblasts, and small lymphocytes and plasma cells obviously lack a functioning system of respective phosphokinases, since these cells incorporate ^{3}H-Tdr into the soluble fraction, if at all, at a negligible rate compared to those cells known to be capable of DNA synthesis. These latter cells incorporate ^{3}H-Tdr into DNA within minutes and one hour or later after a single injection of the labelled precursor, the radioactivity in the soluble fraction of bone marrow cells contains only about 5 % and less of the radioactivity incorporated into DNA, as seen in Table I and II. Comparable findings, indicative of selective thymidine uptake by cells and of a rapid renewal of the thymidine in the soluble fraction, at most over a period of one cell generation time, were also obtained in a variety of other cell systems [2, 7, 8]. It was shown in mice that as little as 0.05 μM thymidine, i.e. about 13 μg, depresses already the incorporation of the thymidine analogue, 5-iodo-deoxyuridine into DNA, when both precursors are injected simultaneously [7]. In addition, free circulating thymidine does not escape passage through the liver finally, where nearly one half is rapidly catabolized with each passage [8].

Therefore, the rapid renewal of acid soluble thymidine in the proliferating cells during DNA synthesis, the comparatively small amount of thymidine found free in the circulation and in the acid soluble fraction of cells argues that a storage pool for thymidine does not exist, it be then within individual cells between 2 periods of DNA synthesis only. The latter has been indeed observed to occur in *tetrahymena pyriformis* [8]. In fact, a delayed thymidine incorporation from an as yet unspecified

TABLE I. Tritium distribution, 1 hour after injection of ^{3}H-thymidine (in dpm per nucleotide fraction per unit cells)

Saline wash	Acid soluble fraction	DNA
314	66	6,050
176	64	6,470

After washing the cells in buffered saline (Saline wash), the acid soluble fraction was extracted with cold 2 % perchloric acid, and following a treatment with ethanol to remove the lipids, the acid insoluble nucleotides were hydrolyzed in hot 10 % perchloric acid. The radio-activity of this latter fraction is listed here under DNA, on the basis of the specificity of ^{3}H-thymidine incorporation into DNA. (The saline wash represents non-volatile tritium.)

TABLE II. Tritium distribution, 2 days after injection of ^{3}H-thymidine (in cpm per nucleotide fraction per unit cells, or in cpm per μg P-nucleotide = specific activity)

Saline cpm	Acid soluble		RNA		DNA I		DNA II		No. cells × 10^8
	cpm	s. a.	cpm	s. a.	cpm	s. a.	cpm	s. a.	
512	144	18.9	360	14.8	37,356	352	2,192	344	1.41
438	141	22.9	411	21.6	33,486	463	1,328	440	1.10

The nucleotide fractions were obtained, after washing the cells in buffered saline, according to a modified Schmidt-Thannhauser method, and the μg P-nucleotides were calculated from spectrophotometric measurements with correction for contaminants from protein (Tsanev and Markov, *Biochim. Biophys. Acta*, **42** : 442 (1960)). The saline wash lists non-volatile tritium. DNA II is the fraction following DNA I, it indicates the residual tritium not removed with DNA I from the DNA-protein complex.

storage, if it occurs, must be comparatively negligible small per total organ, and should become exhausted over a period of one cell generation time.

The recognition of this situation is important for the assessment of the data presented next.   •

2. PHYSIOLOGICAL REUTILIZATION OF THYMIDINE OR ITS NUCLEOTIDES

Measurements of DNA renewal per total bone marrow over a period of 9 to 11 days after a single injection of labelled precursor yields, following a plateau of about 24 hours duration [10, 11], an exponential regression of DNA specific activity,
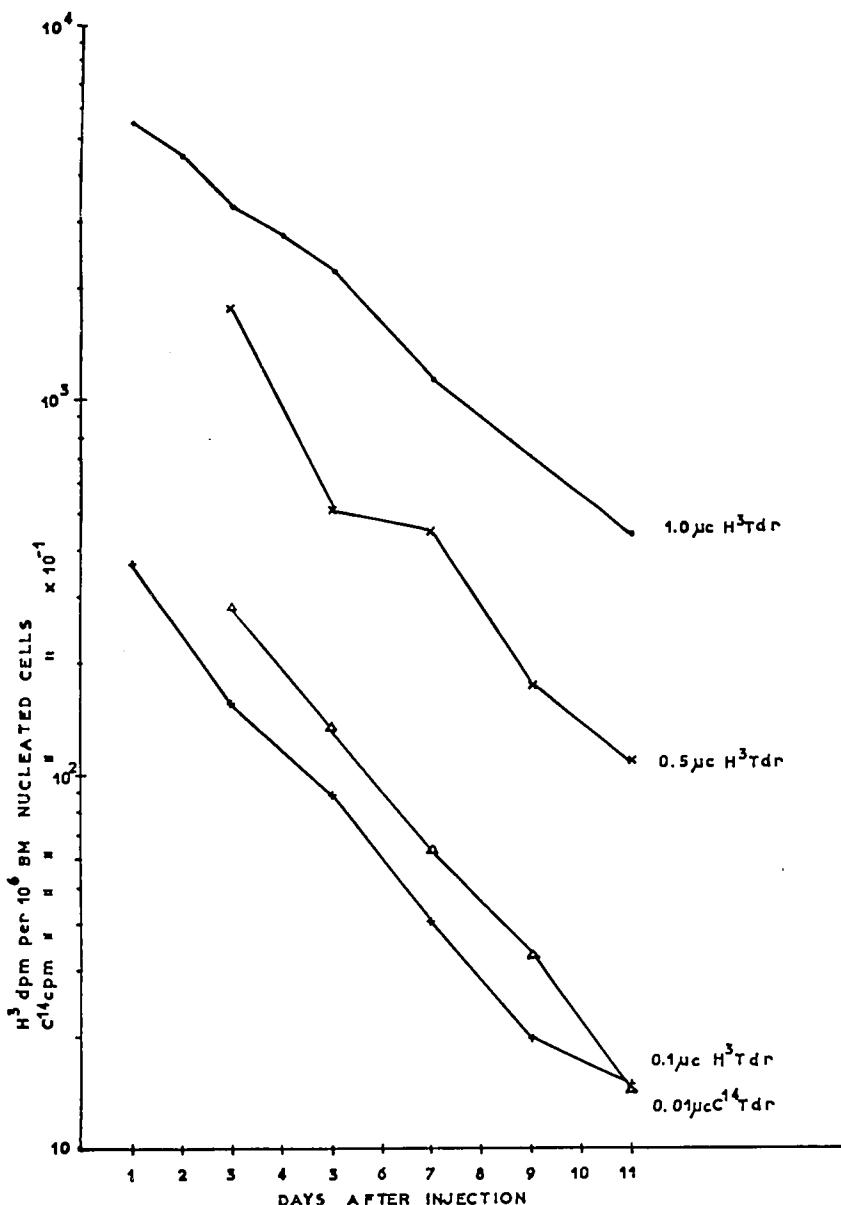
FIG. 1. Regression of radioactivity bound to the acid insoluble fraction (DNA) of 10⁶ nucleated bone marrow cells, after injection of ³H-thymidine or ¹⁴C-thymidine in various doses per gram body weight.

The standard error of injection of tracer and of nucleic acid extraction was below 8 %, as determined in separate experiments on 16 animals in 4 groups.

The counting error in above experiments remained below 4.4 % except for the ¹⁴C values at day 9 and 11, where the error was 9 % and 12 % respectively; and for the ³H value (after injection of 0.1 μC/g body weight) the error was 10 % at day 11.

as shown in Fig. 1 and 2. The results are different, however, according to the type and dose of labelled precursor used. Fig. 1 indicates that after injection of ^{14}C-Tdr the regression of the specific activity proceeds at a rate of 0.347 per day, similar to the regression after injection of ^{3}H-Tdr in a dose of 0.1 and probably also of 0.5 μC per gram body weight. When the ^{3}H-Tdr was however injected in a dose of 1.0 μC per gram body weight, the specific activity declined at a rate of 0.247 per day. With ^{131}I-DU the regression was faster, the daily rate being 0.533 (Fig. 2), regardless whether the measurements were made on isolated cells or on the total bones.

The discrepancy of the data obtained with ^{131}I-DU could perhaps be explained by an inadequacy of ^{131}I-DU as tracer in these experiments. However, it was indicated
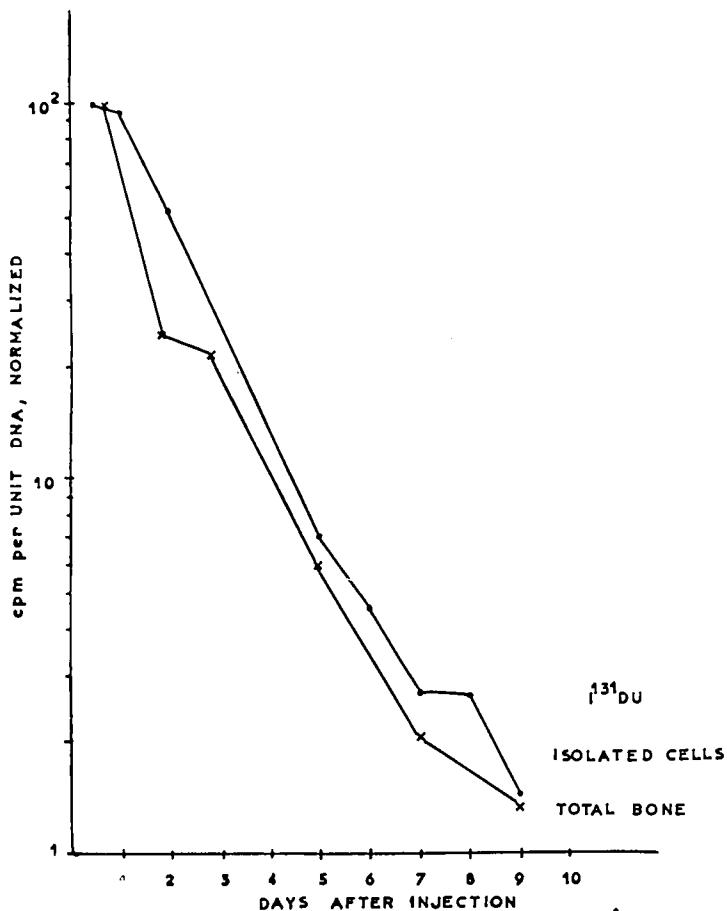

FIG. 2. Regression of ^{131}I bound to the acid insoluble fraction of 10^6 nucleated bone marrow cells or bound to the total bones (2 femurs and 2 tibiae) after injection of ^{131}I-deoxyuridine. The data are normalized to the starting value.

The counting error of the bones remained below 3 %. The counting error of the acid insoluble fraction remained below 2.5 % except for the values on day 5 : 8.5 %, on day 6 : 9 %, on day 7 : 11 %, on day 8 : 12 %, and on day 9 : 14 %.

in confirmation of data in the mouse [7, 12] that also in the rat bone marrow IDU is a specific precursor for DNA [4]. Furthermore, ^{131}I is obviously retained on the native DNA in viable cells, be they proliferating rapidly [4, 13] or be they in a non-proliferating "resting" stage [12].

While 5 to 10 % of IDU injected into mice is retained in the DNA [7] thymidine is known to be utilized more efficiently. Thus in the rat [14] as in humans [15] about one half on the injected precursor is retained while the rest appears catabolized. The relatively poor utilization of IDU for DNA synthesis appears therefore the decisive metabolic property which allows to argue that IDU is as such also poorly reutilized [7, 10]. Indeed, reutilization of IDU in comparison with thymidine after cell death, induced by X-rays, was practically excluded [12].

Toxicity of ^{131}I-DU at the low level used here, may hardly be invoked to explain the data. Thus it was shown that IDU, labelled with ^{125}I, and injected in an amount about 10 times larger but with a specific activity yielding about the same accumulation of radiation dose of 3-6 rads, as in the present experiments with ^{131}I-DU, did not change the number of cells in mitosis significantly 1 hour, 1 day, and 3 days after injection [4]. Hence, a lasting disturbance of cell proliferation was not manifest.

The discrepancy of the regression curves obtained with ^{131}I-DU and with ^{14}C-Tdr, or ^{3}H-Tdr in low dose, must therefore indicate that the ^{14}C labelled DNA, or ^{3}H-DNA after injection of low dose of ^{3}H-Tdr, does not indicate the true renewal but the net renewal in the organ of DNA units, some of which must be reutilized in the physiological condition. The rapid cell turn over in the bone marrow with many nuclei of red cell precursors being phagocytized in the marrow [16] could offer sufficient DNA for catabolism and for supplying thymidine for reutilization. Whether the reutilization pathway involves nucleotides and/or simple thymidine is not clarified, but evidence has been given in favor of the latter [7, 17, 18, 19]. In addition, reutilization from migrating cells may occur, even if only about 1 leukocyte migrates into the parenchyma of 500 cells per hour from the vascular bed in rat bone marrow [20].

The magnitude of the physiological reutilization pathway of the labelled thymidine or its nucleotides may be estimated from the present data, which are combined and normalized in Fig. 3. With 0.533 being the daily rate of decline of the ^{131}I specific activity, and 0.347 being the respective value for ^{14}C-specific activity or for ^{3}H-specific activity in low dose, the difference of 0.186 per day indicates the approximate amount of ^{14}C, or ^{3}H, involved in reutilization. Hence 0.186 of 0.533, i.e. about 35 %, of the label that leaves per day the bone marrow with outflowing cells or is involved in DNA catabolism, is replaced by reutilization of tracer from within the marrow or by other possible routes.

The fact that all regression curves obtained adhere to a simple exponential function between day 1 and 9, attests the non-uniformity of cellular proliferation, since otherwise, due to the discrete labelling pattern of the various types of bone marrow cells, the decline of the DNA specific activity should be stepwise.

It must be pointed out also that the specific activity of ^{14}C and of ^{3}H in low
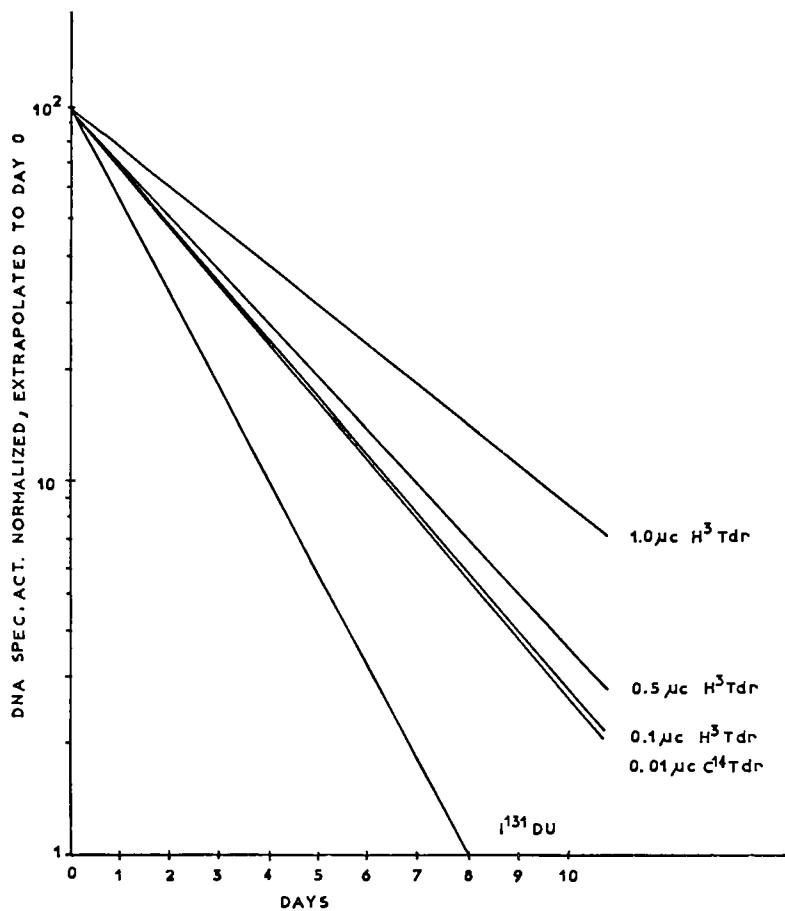
FIG. 3. The exponential parts of the regression curves presented in Fig. 1 and 2 are extrapolated to day 0 by a straight line to best fit the data and are normalized to the starting value. The regression coefficient ($= 0.693$/half-time) is :
0.533/day for ^{131}I,
0.347/day for ^{14}C and for ^{3}H (dose 0.1 μC/g),
0.247/day for ^{3}H (dose 1.0 μC/g).

dose, declines with time in parallel. This indicates the lack of a net effect of tritium in isotopic substitution in the reutilization pathway. Hence, either there is no enzymatic isotope effect at all during the various conversions, or an effect during enzymatic break down is compensated by a reverse effect during another reaction, perhaps during DNA synthesis.

3. TOXICITY OF TRITIUM RADIATION

While the discrepancy between the data from ^{131}I-DU and from ^{14}C-Tdr may be explained by reutilization of the latter, the delayed regression of the ^{3}H-specific

activity after injection of 1 μC ³H-Tdr must be explained by toxic effects from the tritium incorporated in the cell nucleus. Metabolic effects of the thymidine can be excluded, since the amount of precursor remained within the range of true tracer conditions, indicated by the direct proportionality between amount injected and amount incorporated (Fig. 1). The effects observed may be assumed rightly to be due mainly to the beta radiation [21]. Consequently, the rate of cell proliferation may have become altered, or the thymidine reutilization pathway may have become increased from selective killing of a few heavily labelled cells, or DNA repair *in vivo* occurred with subsequent increased availability of DNA units for reutilization by viable cells, or a combination of these is possible. One may calculate, for example, that the DNA-thymidine of a few most heavily labelled cells, totally reutilized at any time later than 24 hours after injection of the tracer, may account for the 30 % reduction in the rate of regression of the specific activity of DNA in the total organ. A small number of cells killed selectively, however, is hardly detectable by ordinary cytological evaluation. Indeed, no clear evidence of cell death was present neither in the bone marrow smears nor in the appropriate sections. If cell death would be the major factor, the DNA specific activity should regress rather at an accelerated than at a delayed rate. It was observed, however, as indicated in Fig. 4, that the number of cells in mitosis dropped abruptly between 12 and 18 hours after injection of ³H-Tdr in a dose of 1 μC per gram body weight.

The mitotic event is known to be a most sensitive indicator of radiation insult, and as little as 5 rads from X-rays caused in skin within 1 hour a temporary arrest of cell proliferation prior to mitosis [22]. The recovery was complete 1 hour later. Because of the sensitivity and rapid response of the mitotic index as parameter of effect of sublethal radiation dose it is possible to deduce from the present data an effective dose delivery time for those cells actually involved, i.e. which are most heavily labelled. Consequently a probable minimum dose delivery time of 12 to 17 hours, perhaps 15 hours, is assumed to be correct.

The question of how much radiation was absorbed by those cells affected is difficult to answer, and the problems associated with micro-dosimetry from tritium incorporated in the cell nucleus have veen discussed recently [21]. Table III lists the relative labelling intensities of the different cell groups in terms of autoradiographic grain counts, and it is obvious that their range in the various cell categories deviates to multiples of the mean value. From the average grain count per total marrow, from the respective labelling index, and from the disintegrations of tritium per number of bone marrow cells at 1 hour after injection of the tracer a mean autoradiographic efficiency factor can be determined, knowing the autoradiographic exposure time necessary to produce the grains. This is summarized in Table IV. Thus 16 to 17 disintegrations are necessary in this experiment to yield 1 autoradiographic grain on the average for the total marrow population. Differences of this value are expected, of course, for single cell types due to changing degrees of self-absorption of the tritium-beta.

On the basis of above calculation for the average bone marrow cell it is estimated

TABLE III. Grain counts per labelled cells, at average with the respective minimum and maximum values, per cell group. The appropriate labelling indices are listed also. 1 hour, 1 day, and 2 days after a single injection of ³H-thymidine, 1 μC/g body weight

	1 hour		1 day		2 days	
	% lab.	grains	% lab.	grains	% lab.	grains
Erythroid, total	49	18; 2-97	98	7; 5-14	94	5; 1-16
Normoblasts (large basophilic)		25; 9-51		13; 9-16		6; 1-16
Myeloid, total	20	23; 2-131	54	10; 1-41	85	10; 1-23
Myelocytes		19; 2-62		10; 2-23		9; 1-21
Lymphocytes						
large	80	34; 20-73	94	22; 1-71	100	10; 4-22
medium	34	18; 3-66	71	10; 2-29	79	8; 3-36
small	2.6	7; 3-14	38	7; 2-16	75	5; 1-19
"Naked nuclei"						
large	89	50; 13-150	85	23; 4-74	100	13; 11-18
medium	59	36; 4-77	89	12; 4-21	95	9; 2-21
small	45	31; 4-78	67	11; 2-23	85	7; 2-46
Megacaryocytes, immature . . .	27	106; 51-175	84	30; 8-110	95	18; 4-42
Plasma cells, mature.	0	0	1.9	7; 4-10	11	8; 3-11

TABLE IV. Calculation of autoradiographic efficiency (at average per total bone marrow cell population)

Time	dpm per 10⁶ cells	% lab. cells	dpm per cell	Grains per lab. cell	Grains per lab. cell per min	Disint. per grain
1 hour .	6,000	27	0.022	26	0.0013	16-17
1 day . .	5,450	65	0.0084	10	0.0005	16-17

NTB₂ (Kodak) emulsion was used, the autoradiographic exposure time was 14 days (20,160 min). The bone marrow was examined at 1 hour and 1 day after a single injection of ³H-thymidine, 1 μC/g body weight.

that, with an autoradiographic exposure time of 14 days, 26 grains per average labelled cell represent 1.2 ^{3}H-disintegrations per hour or near 20 disintegrations per 15 hours (decrease of tritium content by cell division ignored for this time period). Because the grain counts (Table III) range as much as 4 fold from the mean value, between 20 and 100 tritium disintegrations per cell nucleus may suffice to cause the observed effect of proliferative arrest or slow down.
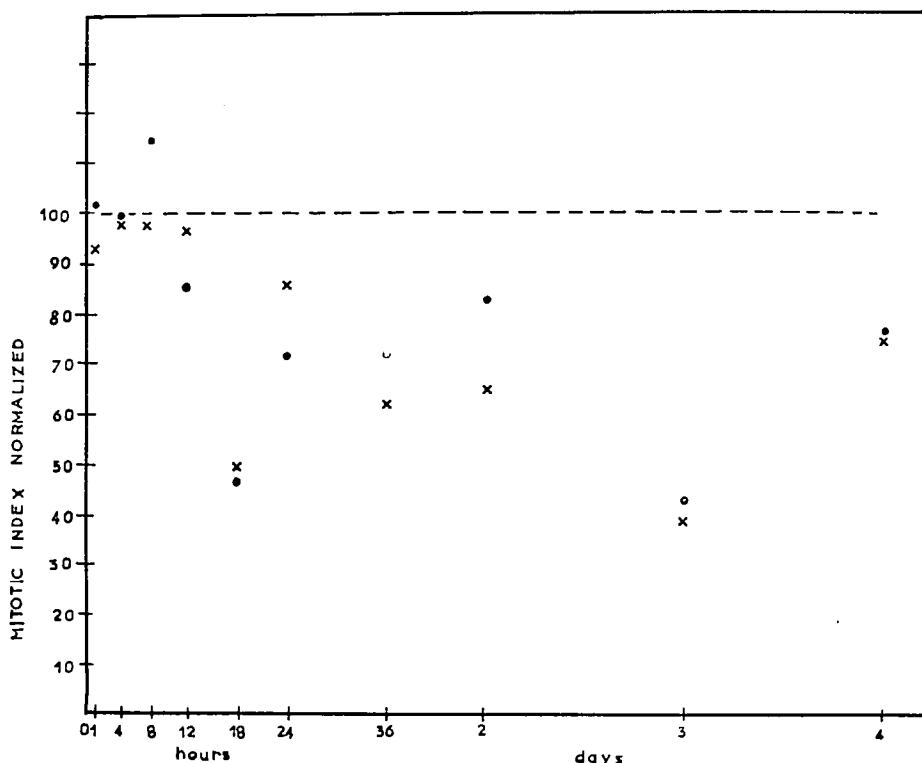

FIG. 4. The mitotic indices in the bone marrow were determined without specification of cell class by counting not less than 6,000 cells per point on different preparations from 1 animal per time interval. After injection of ^{3}H-thymidine, 1 μC/g body weight. The data are normalized to the starting value.

If the average bone marrow cell nucleus is taken to have a diameter of 8 μ, or 270×10^{-12} g, the beta absorption formula may be applied to express the tritium disintegrations per nucleus in the term of rads. Thus

$$\frac{20 \times 5.7 \times 10^{-3} \times 1.6 \times 10^{-6}}{270 \times 10^{-12} \times 100} = 7 \text{ rads}$$

where 20   = tritium disintegrations,

     5.7×10^{-3} = beta energy in MeV, average,

     1.6×10^{-6} = ergs per MeV,

     270×10^{-12} = mass of nucleus, average, in g,

     100   = factor to convert ergs to rads.

This dose needs to be reduced by the amount of beta energy deposited outside the nucleus. This edge effect for a nucleus 8 μ in diameter is approximately 20 % (on the basis of a straight beta trajectory) [23]. Hence the dose becomes maximally reduced to 5-6 rads for 20 tritium disintegrations, and it is 25 to 30 rads for 100 disintegrations. At this dose level proliferative arrest or slow down, indicated by a drop of mitotic indices, has been observed from X-rays [24]. The problem of relative biological effectiveness of the tritium beta in comparison with X-rays in mammals has been discussed elsewhere [21].

The significance of the delayed effect with incomplete recovery up to at least 4 days, as indicated in Fig. 4, is as yet unexplained. Through dose reduction with cell division a delayed effect should hardly be expected, unless one assumes that the cells involved in proliferation slow down at these later times, stem from cells that had preserved more tritium than most other cells proliferating, or a delayed recovery occurred due to accumulation of low dose irradiation from incorporated tritium. Definite effects from ^{3}H-Tdr injected in a dose comparable to that used here, have been observed also in various other mammalian tissues, such as in spermatogonia [25] and in regenerating liver [26].

The toxic effects from tritium radiation in the bone marrow cells presented here advices restraint in the selection of dose for tracer experiments, and it adds, in conjunction with the prove of physiological reutilization of thymidine, to principally reconsider the feasibility of labelled thymidine for long term studies on DNA renewal in mammalian systems.

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DISCUSSION

LAJTHA : The bone marrow works in this way. There is a small stem cells compartment, which is a self-maintaining compartment, allowing flux of cells into differentiation. After 4 to 5 divisions, these cells reach a stage, where there are no more cell cycles, just maturation delay after which the cells leave the bone marrow to go out into the peripheral blood. The population which is synthesizing DNA will take up thymidine, and from this uptake onwards, it will dilute thymidine exponentially. This should give you a slope. This slope will not be disturbed by cells feeding into the stem cells compartment, firstly because the number of these cells is very small compared to the number at any later time, and secondly because these cells will also be labelled and also will divide and will have roughly the same slope with time as the other ones. However, this theoretical slope will be influenced by the population of mature cells, this DNA leaving the bone marrow, so that for this population, the experimental slope should be steeper than the theoretical slope.

The slope should suddenly drop down when the whole population has gone through maturation and come out in the circulating blood. It would then go on to final slope of the stem cells, but this should correspond to a very much lower activity because the number of cells here is very small (less than 1 %). Why don't we see these two slopes ?

FEINENDEGEN : I have two explanations for that effect. The first one is that in red bone marrow only 20 to 30 % of nucleated cells are initially labelled; this is a small number. There are many cells which hang around in the bone marrow and do not become labelled for a long period of time : many of them are lymphocytes.

The other point is that the slope is not exponential in the long run : Hamilton has shown it long ago. The slopes which were demonstrated went over approximately 10 days. Hamilton's data go into weeks and may not be taken as a simple exponential function at the infinite. The reason for this tailing must lie in the kinetic models.

LAJTHA : The iododeoxyuridine is a fairly unnatural precursor. Could't one imagine a slight toxicity of iododeoxyuridine causing a slight cell death and therefore increased turnover ?

FEINENDEGEN : We did not see a toxic effect from IDU on the mitotic indices. I think just that the isotope effect of the iodine is the advantage. Enzymes don't utilize iododeoxyuridine as well as the methylated uridine. This discrimination makes it so suitable to study reutilization.

The amount of radioactivity or iodinated deoxyuridine used in these experiments is very small.

LAJTHA : I don't mean the iodine; even if it were tritium labelled iododeoxy-uridine ?

FEINENDEGEN : That amount is very small and leads to the replacement of about 1 thymidine molecule out of 2×10^5 in the DNA chain, and once incorporated it stays in the native DNA. In the native DNA in the living animal, the iodine just remains in place even for months as commerford has shown.

KAY : I wonder if you discounted the cell destruction from ^{3}H too easily on the absence of reference giving evidence of this. Because I think, the striking thing is the absence with which the reticular endothelium system disposes of a very large amount of cells with very little evidence indeed. It is normally disposing of a lot of normoblast nuclei.

FEINENDEGEN : Our experiments are directed to that question and at the moment, the data only show that, regardless of cell killing, there is a slowdown of cell proliferation. I tried to calculate the degree of slowdown of proliferation from the mitotic index data, assuming that the mitotic time remains stable and the two slopes fit. But I do not think that it is valid to do so because of a number of uncertainties, and it is very possible that some cell killing may have occured, unobserved by the ordinary cytological techniques.

HOLLDORF : You did not add non-labelled material. This explains some of your differences. In the first case your concentration is much lower than in the second case, possibly one enzyme is much more saturated in a pathway than another enzyme in the different cases.

FEINENDEGEN : We have only indirect data to get after that. The effective thymidine pool in the mouse is approximately 12 μg thymidine in the total animal. Now if one takes this as a basis for the rat, it goes to 120 μg for a 200 g rat. We operated far below that level and I indicated in Figure 1 that the amount of tritiated thymidine incorporated reflected directly the amount injected. In other words at those levels we were still in the range of tracer doses.

BRESCIANI : May I ask about these effects on mitotic index. Did you try any dose lower than 1 microcurie per gram ?

FEINENDEGEN : I have no data yet on the dose between 0.5 μC and 1 μC and I have not examined the mitotic index at 0.5 μC. The discrepancy of the regression line initiated the cytological investigation of the preparation after one microcurie of tritiated thymidine, and the only thing we could find there until now was a drop in the mitotic index. I have done some sporadic checks of the 0.5 μC ^{3}H-thymidine and ^{14}C-thymidine labelled cells, and the mitotic indices were within the normal range.

BRESCIANI : Another question is connected with this effect on mitosis by one microcurie dose. Could not there be, at least at the initial part, a G2 effect, which means a blocking of cells in G2 so that mitotic index drops ?

FEINENDEGEN : Yes, I think this indeed happened. Interesting is that the mitotic index, expected to return to the original value, stayed below the normal level.

BRESCIANI : Yes, but then at 4 days it starts going up. I wonder if you have looked after the 4th day to see if there was any overshoot.

FEINENDEGEN : No, I did not yet. If there would be a G2 effect only, which would be temporary, mitotic index would come back but in fact it remained low. Perhaps the cells with less radioactivity incorporated, exerted an effect later than those cells which were heavily labelled. There is, of course, dose dilution by cell division, but there is also dose accumulation and there must perhaps be enough to cause this mitotic index to remain below the normal value.

KRÖGER : Do you know if the iododeoxyuridine has any influence on the phosphorylation of thymidine or TMP, because the main action of the iododeoxyuridine according to Prusoff is the inhibition of the phosphorylation ?

FEINENDEGEN : I think Doctor Schultze should answer this question because she worked in the group who checked the competition between ^{3}H-thymidine and IDU. We did only one experiment; we gave huge amounts of cold thymidine to flood the animal and to flood the pool and gave iododeoxyuridine at various times after the thymidine injection. There is no doubt that the thymidine inhibits the iododeoxyuridine uptake.

GERBER : In answer to a question of Dr. Holldorf about influence of the amount of thymidine given in this replacement, we have only a few data. We have studied in a few cases replacement of thymidine after injection of various quantities of thymine of the same activity level and we do not find a difference in a number of organs. With respect to your observation, whether there is a difference between ^{14}C or ^{3}H we can confirm that we do not find any influence, nor an isotopic effect : the ratio of specific activity of ^{14}C to ^{3}H is the same and we did not only study bone marrow but we studied also liver, spleen, thymus, testis and intestine. We do not find a difference there too.

KRÖGER : You said that in animals there is a reutilization of RNA and DNA. Now it is known from bacteria that polynucleotide phosphorylase can make this conversion to the diphosphates. And i want to know if polynucleodite phosphorylase plays a role in animal tissues too ?

FEINENDEGEN : The only thing I know is that when we inject tritiated cytidine, the overall specific activity of RNA goes down and the DNA runs parallel. This is of course not representative of the true situation, because if one looks at single cells, RNA renews and DNA does not. The fact that the regression curves for the total tissue are parallel is just fortuitous.

Because of RNA renewal in single cells while DNA is stable RNA reutilization exceeds that of DNA from dead cells, and in the overall heterogeneous cell population, the two curves are parallel.

In fact, I think the two main sources of the DNA thymine of bone marrow are DNA and reutilization from DNA.

LETHALITY FROM ^{3}H-NUCLEOSIDES
INCORPORATED IN MAMMALIAN CELLS

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The survival kinetics of cultured chinese hamster cells (strain CHEF-125) after incorporation of tritiated thymidine or tritiated uridine, were studied by Marin and Bender (1963). These authors compared the lethal efficiency of the two tritiated nucleosides on the basis of their geometrical distribution within the cell. The conclusions reached from that work were the following :

1) Lethality was closely dependent on the amount of incorporated tritium;
2) The basic survival kinetics for ^{3}H-thymidine (i.e. those for "first generation killing") conformed to those obtained with X-radiation, and fitted the expression $S = 1 - (1 - e^{-kd})^n$, with $n = 1.9 \pm 0.4$;
3) A rough estimate of the dose-rate for internal irradiation from tritium incorporated in the nucleus, gave a value of about 130 rads/hour at the 10 % survival level. The corresponding total dose delivered to a cell and its descendants, appeared to be higher than the dose needed to give the same survival under acute exposure:
4) Tritium proved more effective when incorporated in the form of ^{3}H-thymidine than in the form of ^{3}H-uridine, although some of the tritium incorporated in the cytoplasm (as ^{3}H-uridine was given to the cells), did appear to contribute to lethality.

The preliminary results of a slightly different approach to the same problem will be presented here.

A subline of strain BHK-21 of syrian hamster fibroblasts (Stoker and Macpherson, 1964) was used. In our laboratory this line displays a generation time of 8-10 hours and a plating efficiency averaging 50 %. Modified Eagle's medium (the content of aminoacids and vitamins is increased fourfold) supplemented with 10% undialized calf serum, was routinely used.

The experiments were carried out as follows. Parallel cultures were exposed for 1 hour to various levels of ^{3}H-thymidine or ^{3}H-uridine in the medium, and then incubated in the presence of cold thymidine or uridine as chasers. Four hours later the cells in mitosis were selectively harvested according to the method of Terasima and Tolmach (1961). From each cell suspension obtained in this way, samples were drawn and plated to measure survival. The remaining cells were extracted with boiling 10 % TCA for 20 minutes, and the specific activity of such extracts was measured and expressed as the ratio cpm/OD_{260}. It had been previously determined that the distance between the midpoint of the DNA synthetic period (S) and mitosis,

was about 4-5 hours in this strain. The cells harvested, therefore, were almost 100 % labelled both when they had been incubated with ³H-thymidine and when they had been incubated with ³H-uridine. The two cell populations were quite comparable : internal irradiation would start at the same time in both of them, and all the cells would undergo division (which is the main cause of reduction of the radiation dose per cell) in synchrony.

The results shown in Fig. 1 confirm those previously obtained on strain CHEF-125, where autoradiographic grain counts, rather than the specific activity of TCA extracts, had been used to give an estimate of the radiation dose delivered to the cell. In both cases, incorporated ³H-thymidine appears to be more effective than ³H-uridine in causing cell death.
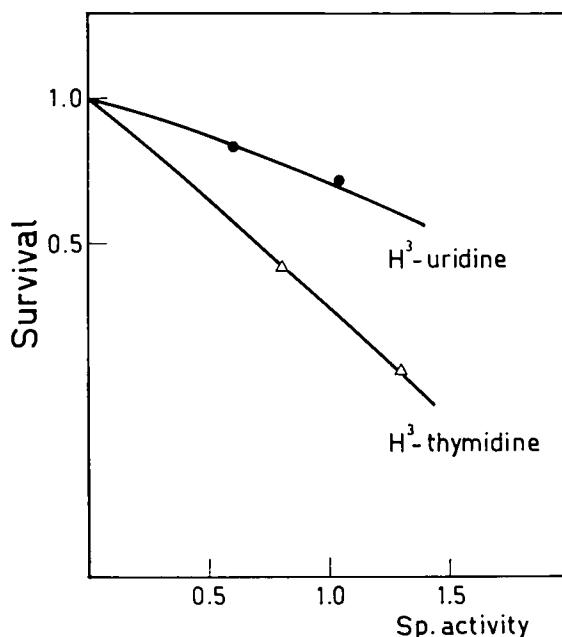
FIG. 1. Per cent survival of cells as a function of ³H-thymidine or ³H-uridine incorporation. The specific activity of whole-cell TCA extracts is reported on the abscissa.

The simplest explanation of this finding is that in the former case all the radio-activity is permanently confined, in the form of DNA, to the most sensitive part of the cell — the nucleus —, whereas in the latter case most of it, after being incorporated in the nucleus, moves to the cytoplasm as ribosomal RNA (Feinendegen and others, 1960).

The following experiment was designed in order to add indirect support to the above interpretation. In this case some of the cells harvested and plated as described above, were immediately incubated at 37° C, and others were kept for various lenghts

of time at 20° C, and *after* put back in the incubator. All the cells had been exposed to the same concentration of ³H-thymidine or ³H-uridine. Colony counts were carried out after 6 days of incubation at 37° C in all cases. During the period spent at 20° C the cells survived without dividing. As soon as they were put back in the incubator, they would resume multiplication without any appreciable loss, as long as the time spent at 20° C had not exceeded 40-50 hours.
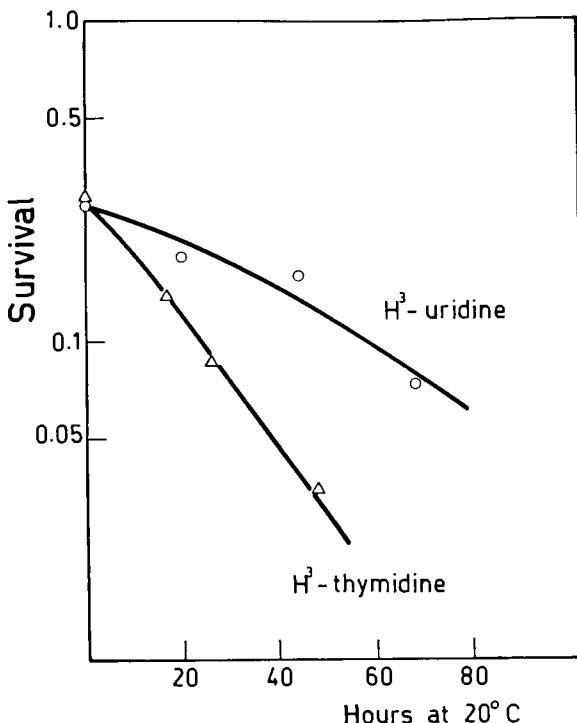

FIG. 2. Per cent survival of cells exposed to a constant dose of ³H-thymidine or ³H-uridine, as a function of the time spent at 20° C after incorporation. The values on the abcissa indicate the number of hours for which the cells were kept at 20° C after plating, and before being put back at 37° C.

By keeping the cells from dividing, the reduction of the radiation dose per cell due to segregation of tritium in the two daughters at division, is prevented. If one assumes, however, that only tritium incorporated in the nucleus is effective in killing the cell, one may expect that by delaying division lethality should be increased *more* in ³H-thymidine labelled cells than in ³H-uridine labelled ones, where most of the tritium goes to the cytoplasm after a few hours anyhow.

The results reported in Fig. 2 indeed show that by increasing the period of storage at 20° C, lethality due to the incorporation of ³H-thymidine is enhanced more than lethality due to the incorporation of ³H-uridine.

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DISCUSSION

ALTMAN : I presume that the labelling of the uridine is uniform with respect to tritium. How is the thymidine labelled ?

MARIN : It was methyl labelled thymidine, and uridine was uniformly labelled.

LAJTHA : It was not quite clear to me. Could you explain again in your last slide the difference of the cause of the difference for the initial 0 time values ? In your last slide, you are plotting the survival against exposure at room temperature and already at 0 hour exposure at room temperature, there is quite a difference between the two experiments in the survival values.

MARIN : There are two different incorporation levels. There are two different experiments, where we used two different levels of thymidine and uridine in the medium.

LAJTHA : Which means that your cells have received a considerable dose of radiation for a considerable time before explantation for plating. Much radioactivity coming into the cell requires a certain time to accumulate a certain dose.

MARIN : I labelled the cells at the given moment. The cells go to division (that is about 5 hours). At this time I plate the cells, then the cells start to grow and make colonies and, after 5 days, I count the survivals in one case.

LAJTHA : Is that the 0 time on the last curve ?

MARIN : No, the 0 time is always the same on all curves, the only difference is that in some cases, just after plating cells, I keep them at room temperature for different lengths of time, so here there is an added time at room temperature which varies.

LAJTHA : That is exactly what you are marking at the blackboard, the 0 time for room temperature is preceeded by 5 hours period at 37º C. What is the estimated dose rate at room temperature in case of thymidine?

MARIN : I did not calculate this in the experiment.

LAJTHA : The dose rate is sufficiently low, you would expect in any case a so called single-hit type of curve.

With use of any external radiation X rays or γ rays (which is so called one shot) this is a high dose rate, giving a typical two or three extrapolation numbers. If we give the same dose in a protected fashion at sufficiently low dose rate extrapolation numbers go down until it is one. And that is the nature of the survival curve. You are in fact dealing with the single hit damage only. There is no chance for a second hit.

ISOTOPIC DISCRIMINATION BETWEEN ^{3}H AND ^{14}C
IN VITRO DURING ENZYMATIC PHOSPHORYLATION
OF LABELLED THYMIDINE

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It is well known that tritium (^{3}H) may be responsible for rather important isotopic effects (Broda [1]; Isbell *et al.* [2]; Klein *et al.* [3]).

During a study of the effects of X-irradiation on thymidine and thymidylic kinases in regenerating rat liver (Goutier *et al.* [4]), we noticed that the proportion of thymidine phosphorylated by a liver extract *in vitro* was not the same for ^{3}H or ^{14}C labelled thymidine (TdR). The present work started from that observation. A preliminary communication has been published (Baugnet-Mahieu *et al.* [5]).

TECHNIQUES

1. LIVER EXTRACTS

Male Wistar rats of 180 g are partially hepatectomized according to the technique of Higgins and Anderson [6] and sacrificed by decapitation 36 to 40 hours later. Regenerating livers are perfused in ice cold 0.9 % NaCl, then homogenized in 2 vol cold distilled water pH 7 in a Potter tube. The 30 % homogenate is spun at 300 000 g for 60 min; the supernatant (20 to 30 mg proteins per ml) is used as active extract.

2. ENZYME PURIFICATION

TdR-kinase has been purified 150 to 200 fold from regenerating rat liver according to the technique of Weissman *et al* [7]. TMP-kinase activity is lost during the purification procedure.

3. ENZYMIC ASSAYS

The composition of the assay mixture is the following, in a final vol of 0.7 ml :

Tris-HCl buffer pH 7.9	55 μ moles
ATP	5 μ moles
MgCl$_2$	2.5 μ moles
TdR	30 to 40 mμ moles
	1 μCi ^{14}C or 8 to 10 μCi ^{3}H
Active extract	2 to 5 mg protein
or purified kinase	30 to 600 μg protein

After usually 30 min incubation at 37° C, the reaction is stopped by cooling the mixture and adding 0.1 ml $HClO_4$ 10 N. The cold acid-soluble fraction is neutralized with 7 N KOH and submitted to chromatography on column or paper. The enzymatic activity is expressed from radioactivity measurements as the percentage of TdR converted to TMP, TDP and TTP, or as the amount of phosphorylated products formed.

Proteins are determined with the Folin-Ciocalteu reagent.

4. Chromatography

The chromatographic techniques have been described elsewhere and the validity of the routine planimetric measurements of the radioactivity peaks obtained in an automatic Baird-Atomic Scanner have been assessed by comparing results obtained by four different methods [8] : (a) planimetry on paper records; (b) rates of radio-activity recorded with the help of a printer connected to the paper chromatogram-scanner; (c) radioactivity measured by liquid scintillation after cutting the paper strip into pieces; (d) radioactivity of column eluate portions measured by liquid scintillation.

5. Products used

All non labelled nucleotides were purchased from Sigma Chemical Company.
— Tdr-6-^{3}H — specific activity 3 Ci/m mole was supplied by Schwartz or Amersham,
— Tdr-methyl-^{3}H — specific activity 2.7 Ci/m mole, from Amersham,
— Tdr-2-^{14}C — specific activity 30 mCi/m mole, from Amersham,
— Tdr-methyl-^{14}C — specific activity 1.7 m Ci/m mole was synthesized at the CEN, Mol, by Dr. M. Winand.

Results

Table I gives the percentages of phosphorylation of TdR obtained with 4 different liver extracts. In each case, equal amounts (33 mμ moles) of both labelled TdR have been used.

The enzymatic activity varies from one extract to the other according to individual and seasonal factors [9]. Nevertheless, in all cases the phosphorylation of 6-^{3}H-TdR to TTP is only 0.6 to 0.8 that of 2-^{14}C-TdR. The same difference also exists when the level of phosphorylation of TdR is measured in relation to the amount of substrate, or to the amount of phosphate donor, ATP (see [8]), or to the time of incubation (Fig. 1).

As seen in Table I and Fig. 1, the difference between ^{14}C and ^{3}H compounds is greater at the triphosphate level than at the monophosphate level. In cases where ^{3}H-TMP is produced in higher amounts than ^{14}C-TMP, it must be realized that more ^{3}H-TdR than ^{14}C-TdR remains at the end of the incubation and that, therefore, the equilibrium constant for TMP production, given by the ratio $\dfrac{TMP}{TdR}$ is higher for

^{14}C-TMP than for ^{3}H-TMP. Results with purified kinase preparations will substantiate this.

The autoradiolysis products of ^{3}H-TdR samples, separated in the solvents used by Apelgot and Ekert [10] and by Evans and Stanford [11], represent between 10 and 20 % of the total radioactivity. Since, in our enzymatic assays, the ^{3}H-TdR is diluted

TABLE I. Percentage of TdR phosphorylated by four aqueous liver extracts

Incubation 30 min
TdR-2-^{14}C   33 mμ moles — 1 μC
TdR-6-^{3}H   33 mμ moles — 10 μC

	TdR phosphorylated (%)			
	6-^{3}H-TdR		2-^{14}C-TdR	
	TMP	TDP + TTP	TMP	TDP + TTP
1	4.0	38.3	4.2	57.2
2	4.7	17.1	3.4	28.9
3	17.4	42	11.2	65.7
4	13.2	34	11.8	38
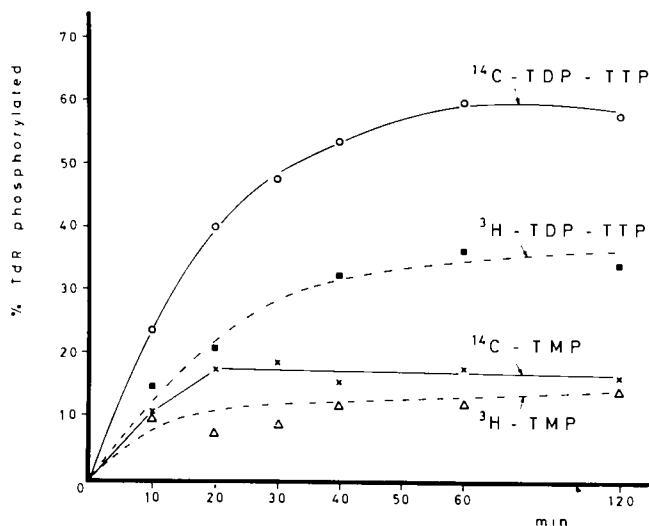
FIG. 1. Phosphorylation of TdR by a rat thymus extract, prepared in the same way as liver extract, in relation to time of incubation.
2-^{14}C-TdR : 33 mμ moles, 1 μC,
6-^{3}H-TdR : 33 mμ moles, 10 μC.

10 times with cold TdR, the actual small decrease in the amount of ³H-TdR in the stock solution due to the presence of radioactive degradation products cannot have any influence on the phosphorylation rate.

But degradation products might influence the enzyme activity. This possibility was tested by submitting to the action of the same liver extract 50 mμ moles of ³H-TdR and ¹⁴C-TdR separately and in mixture. The chromatogram paper strips are cut in squares and the radioactivity is measured by liquid scintillation at two different high tensions (800 V and 1,000 V) in a Packard Tri-Carb counter Mod 314. With the help of internal standards, the radioactivity due to ³H and ¹⁴C can be measured in a mixture of both isotopes. Fig. 2 shows that the phosphorylation of ¹⁴C-Tdr is the same in the presence and in the absence of ³H-TdR. Therefore, kinase activity does not seem to be inhibited by degradation products from ³H-TdR.

The same isotopic discrimination between ³H and ¹⁴C is also observed with methyl-labelled TdR (Table II). High amounts of ¹⁴C-methyl-TdR had to be used in view of the low specific activity of the compound.
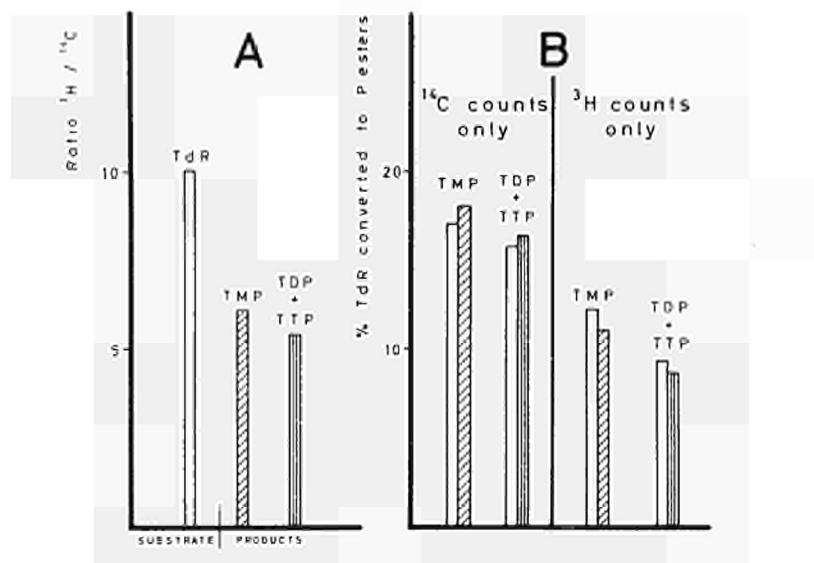

FIG. 2. Phosphorylation of 10 μCi ³H-TdR and 1 μCi ¹⁴C-TdR (50 mμ moles) by a liver extract

A. The two thymidines mixed together in the same test tube. Isotopic ratio in the substrate (TdR) and in the products (TMP and TDP ÷ TTP).

B. Proportion (in %) of TdR converted to P esters.

White bars : ¹⁴C-TdR incubated alone (left) or ³H-TdR incubated alone (right).

Hatched bars : ¹⁴C-TdR and ³H-TdR incubated together; left : ¹⁴C counts only; right : ³H counts only.

TABLE II. Amounts of TMP and TDP + TTP formed (in mμ moles) by two regenerating liver extracts, with TdR labelled on the ring or on the methyl group. Incubation : 30 min

	1				2				
Position and nature of label . .	2-¹⁴C	¹⁴CH₃	6-³H	CH₂³H	2-¹⁴C	2-¹⁴C	¹⁴CH₃	6-³H	CH₂³H
mμ moles TdR incubated . . .	34	754	32.5	33.7	34	384	377	32.6	33.7
mμ moles TMP	2.2	17.4	1.75	2.4	2.6	6.9	6.0	1.2	1.75
TDP + TTP .	12.5	15.1	6.8	7.8	8.5	11.5	11.7	6.2	5.9

PURIFIED KINASE PREPARATIONS

The kinetics of TMP formation are shown in Fig. 3 where different amounts of TdR kinase have been used. Two main differences between ¹⁴C and ³H-TMP are noticeable : the maximum amount of TMP formed is smaller for ³H-TMP and the rate for reaching the plateau region is also lower for ³H-TMP than for ¹⁴C-TMP. This last fact is clearly seen in Fig. 4 where the time taken to reach the plateau (calculated from the initial velocity) is plotted against the reciprocal of the amount of enzyme added. Two straight lines are obtained, with different slopes for ¹⁴C-TMP and ³H-TMP.
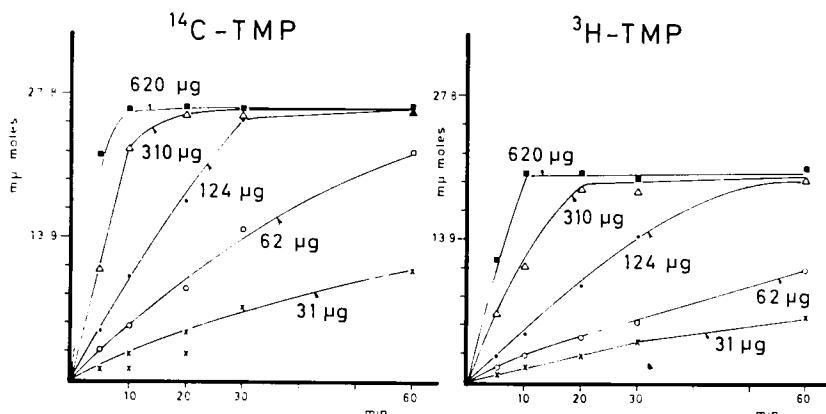
FIG. 3. Phosphorylation of 27.8 mμ moles of ¹⁴C-TdR and ³H-TdR by different amounts (in μg protein) of the same purified kinase preparation.
Abscissae : time of incubation,
Ordinates : mμ moles TMP formed.
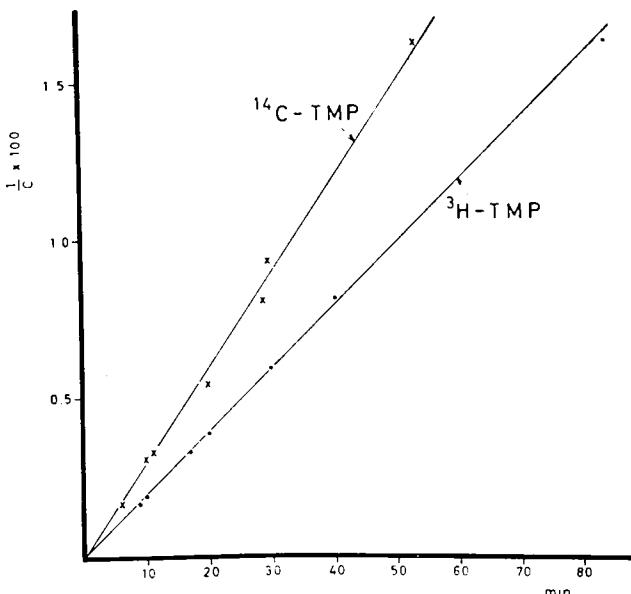
FIG. 4. Phosphorylation of 27.8 mμ moles of ¹⁴C-TdR and ³H-TdR by different amounts of purified kinase.

Abscissae : time taken to reach the maximum phosphorylation level (calculated from the initial velocity).

Ordinates : reciprocal of the enzyme protein amount.

DISCUSSION

In a private communication, Shooter [12] recently confirmed the differences we have described between phosphorylation of ¹⁴C and ³H-TdR.

As we have shown, autoradiolysis products of ³H-TdR do not seem to interfere with the enzyme activity.

TdR is catabolized mainly by the liver (Fink *et al.* [13]; Rubini *et al.* [14]), but since the same isotopic discrimination is observed with thymus extracts (Fig. 1), which do not catabolise TdR, and with purified kinase preparations, it does not seem that catabolism of TdR plays any role in the discrimination observed. If catabolism occurs during incubation with liver extracts and results in some loss of total radioactivity, it seems that the specific activity of TdR is maintained. This is consistent with the observations of Evans [15] and of Winand and Gouverneur [16] who did not find any decrease in the specific activity of TdR during transribosylation of ³H-thymine incubated with a rat or horse liver extract.

There remains the possibility of an isotope effect. As is expected from the theory, indeed, the heavier isotope reacts more slowly than the lighter one (see Broda [1]). This is, of course, particularly marked for hydrogen isotopes, especially in cases where hydrogen removal is involved in the chemical or enzymological reaction (Broda [1]).

The case of TdR-phosphorylation is different, because the enzymatic reaction takes place on C 5 of the deoxyribose ring, not on C 6 or CH_3 of the pyrimidine ring where the tritium is located.

The existence on the active center of TdR kinase, of two different binding sites, one for the esterification of deoxyribose and one for the thymine moiety (Ives *et al.* [17]) could provide a possible hypothesis of the mechanism of the isotope effect. One might consider, although very speculatively, that the presence of ^{3}H on the thymine moiety interferes with the binding of the thymine to or with its release from the active center of the enzyme.

It is interesting to note that the isotopic discrimination described here *in vitro* does not seem to show up in whole animals. Indeed, neither in the incorporation rate of thymidine into DNA (Gerber [18]), nor in the reutilization of thymidine from labelled DNA (Feinendegen [19]) can one detect any significant difference between ^{3}H or ^{14}C labelled TdR. But, of course, there is a vast difference between conditions of *in vitro* incubations and normal working conditions of an enzyme *in vivo*. In the first case, excess of substrate is usually supplied for the sake of an easier study of enzyme kinetics, and the final products of the reaction accumulate in the test tube. In a living cell, on the contrary, substrate is handled as soon as it is produced and final products themselves are taken up at once for DNA synthesis without accumulating (except in pathological conditions where DNA synthesis is impaired). Moreover, DNA labelling experiments in mammals utilize relatively small amounts to TdR.

Therefore, it is improbable that the high substrate/enzyme ratios at which isotopic discrimination is observed *in vitro*, will be met *in vivo*.

The only case where the possibility of an isotope effect of ^{3}H *in vivo* has been suggested is the observation of Rubini *et al.* [20] that bone-marrow cells in culture take up only a limited amount of ^{3}H-TdR during a rather short time into their DNA, although in this case other explanations are also proposed.

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DISCUSSION

EVANS : I would just like to ask Dr. Goutier whether he would not consider using thymidine labelled in the methyl group for testing the hypothesis as to whether the 6 position is involved in this reaction.

GOUTIER : We tried it, but the trouble is that the specific activity of methyl-^{14}C thymidine is very low so that we must use large amounts of it for the experiments. This means that the phosphorylation will also be low : only 6 to 7 % of methyl-labelled thymidine is phosphorylated, versus 40 % for thymidine labelled in the 6th position. But, despite this source of error, we observe the same difference between ^{3}H and ^{14}C when the label is on the methyl group.

APELGOT : Si c'est un effet isotopique par suite de la position en 6 du tritium, c'est la thymidine marquée sur le méthyle qu'il faudrait comparer à la thymidine marquée en position 6 dans les mêmes conditions.

GOUTIER : La thymidine 6 tritiée et la thymidine méthyle tritiée donnent les mêmes résultats, à quantités égales. Il est possible que le groupe méthyle soit très important dans la formation du complexe enzyme substrat parce que la kinase de la thymidine est un enzyme très spécifique de la thymidine et le groupe CH3 est très spécifique de la thymidine également.

SMITH : Can you give me very briefly some ideas of how you prepared the ^{14}C methyl labelled thymidine ?

WINAND : Il existe différentes méthodes et nous avons utilisé la méthode qui consiste à partir de désoxyuridine et à y greffer le groupement méthyle par l'intermédiaire de la formaldéhyde. Par réaction de la désoxyuridine avec la formaldéhyde en milieu alcalin, vous obtenez l'hydroxyméthyle désoxyuridine que l'on peut réduire par hydrogène pour obtenir la thymidine. Dans ce cas, vous avez un marquage sur le méthyle dû au greffage de la formaldéhyde sur la désoxyuridine.

Une autre méthode, qui est d'ailleurs tout à fait similaire, consiste à préparer la chlorométhyle désoxyuridine, c'est-à-dire en traitant la désoxyuridine par la formaldéhyde en présence d'acide chlorhydrique. La réduction de la chlorométhyle désoxyuridine donne exactement le même produit.

APELGOT : Pour revenir à l'effet isotopique du tritium, il faudrait peut-être essayer de faire les mêmes expériences avec de la thymidine marquée avec du deutérium, parce que l'effet isotopique n'est peut-être pas le même.

PALM : If you have a true isotopic effect, with an excess of your enzyme preparation you should finally get up to the same specific activity at the end of 100 % yield.

I think your data which showed that with a high excess of enzyme you end up with 30 % lower yield for ^3H is not a good indication for an isotopic effect, because you don't loose any tritium.

GOUTIER : We should find the same maximal phosphorylation in all conditions, this is one point which is hard to explain. There is also another difference in the rate of phosphorylation.

LAJTHA : If these results are attempted to be explained on radiation effects, some estimate could be made of the dose involved and similarly a model system of this enzyme in solution could be established on which some electron spin resonance studies could double-check these theoretical dose calculations.

With the excess enzyme you may get a protective effect even though you are not reaching the theoretical 100 % saturation, simply by protecting and radical scavenging.

GOUTIER : We would like to check this theoretical radiation effects by doing an incubation in tritiated water or by trying a larger scale of amounts of ^3H thymidine, let say from 1 to 100 microcuries. This is still a narrow range for detecting radiation effect.

BRESCIANI : I gather from your data that the Michaelis constant increases ? Is that right ?

GOUTIER : We have not calculated the Michaelis constant yet.

SKOELD : If I remember it correctly, Kornberg and collaborators showed a very strong allosteric effect of CDP and nucleoside triphosphates on thymidine kinase from bacterial sources and I wonder whether it is possible that a slight impurity with CDP or nucleoside triphosphate in your thymidine preparations could explain this. I remember you had mixture experiments, but to get an allosteric effect you might need very small amounts of these compounds. From Kornberg's work there were very strong effects on the Michaelis constant for the thymidine kinase. Did you purify your thymidine or is it a commercial preparation ?

GOUTIER : It is a commercial preparation from Amersham.

SKOELD : Were the samples purified before use as substrate in the experiments ?

GOUTIER : No. We did paper chromatography just to check the preparation.

HOLLDORF : According to Kornberg's work, the most effective compounds are deoxycompounds : deoxycytidine triphosphate and deoxyguanosine triphosphate and it is difficult to imagine that in commercial ATP preparations, or that in your assay, these compounds could be formed at such a concentration that it could act as feedback inhibitors. The phosphorylation of the deoxycompounds, deoxycytidine, if there was a small amount in the thymidine, is so low that it should not be sufficient to give this feedback.

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