CNEA
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Department of Radiobiology Progress Report 1975 - 1976

Comisión Nacional
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Dirección de
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Gerencia de
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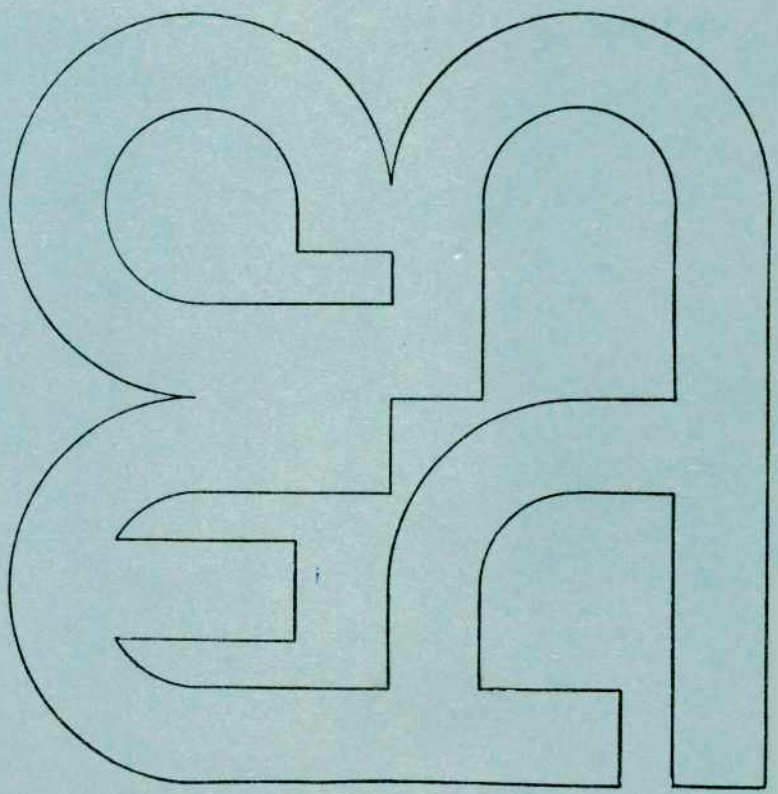

Buenos Aires - March 1977

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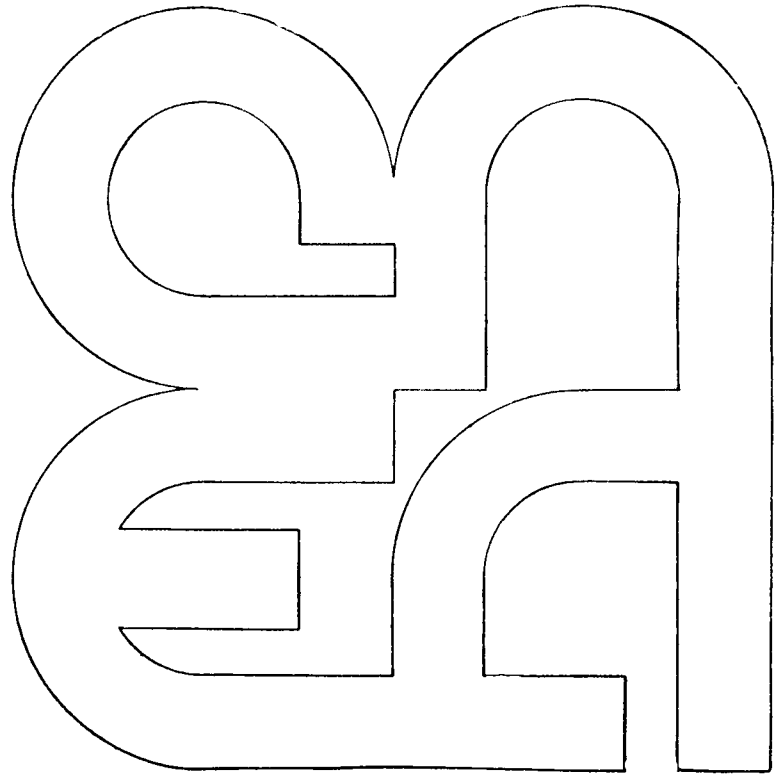

Buenos Aires - March 1977

Introduction

The Department of Radiobiology of the Argentine National Atomic Energy Commission (CNEA) presents in this report a detailed account of the activivities of the last two years. This is the result of a joint effort made by the authors of each paper and by an Institution which guided by a deep sense of responsibility for the future of human society, seeks the continuous scientific progress of the country.

Starting as Department of Biology and Medicine, the present Department of Radiobiology was the origin of many other important scientific activities within and outside this Institution. During the last 20 years, a large number of scientists, now working at other Institutes in this country and abroad, received their initial instruction or advanced their knowledge in the laboratories of our department.

Our scientists have carried out important studies on the mechanisms of radiation damage and radiation repair. The methodology used in these research projects has been also applied in other programs outside the CNEA, for our staff has been frequently asked for advice in handling specialized techniques as autoradiography, chromosome mapping, radioimmunoassay, microspectrophotometry, histochemistry, electron microscopy, etc.

The Section of Irradiation and Dosimetry provided during these years an excellent service for our own staff as well as for scientists of other Institutes.

The same can be said of our Animal Breeding Unit which provides laboratory animals for our own experiments and has been the origin of many animal production laboratories in our country. The outstanding quality of our animals will be further improved in the near future when the new SPF facilities will start the laboratory animal production in the Ezeiza Atomic

Center near Buenos Aires.

My intention not to enumerate the many individuals, who have cooperated in achieving these results, in order not to be unfair through name omission, is interrupted by a new reminder of Andrés Klein-Szanto to deliver the present Introduction. His dedication and enthusiasm as Editor of this Report merit our deep gratitude.

V. L. Orce
Chief
Department of Radiobiology

FOREWORD

The research work carried-out during the last two years, has been devoted, as in previous years, to the study of radiation effects in different biological systems, as well as to developing new techniques which could improve the detection of radiation-induced injury. The employed systems included, microbiological models, insects and mammals, the used techniques varying markedly according to the problems under consideration.

For a better methodological arrangement of this biannual report the activities of the Department of Radiobiology have been schematically classified under the following headings: A) Biochemistry B) Immunology, C) Genetics, D) Microbiology, E) Somatic Effects (Oncology) F) Pathology and G) Irradiation and Dosimetry.

Each of these sections contain several summarized papers, which reflect the principal activities of our laboratories. Those readers interested in individual reports are encouraged to refer to more complete descriptions, as soon as the papers appear in the scientific literature.

The Editor would like to acknowledge the support and comments of Dr. R.L. Cabrini, Head of the Research Management Branch, CNEA, and Dr. V.L. Orce, Chief of the Department of Radiobiology, CNEA, as well as the cooperation of Dr. E. Ventura, Dr. D. Antón, and all the authors of the individual reports, who through their kind effort have made easier the Editor's task. The Editor is also indebted to Miss M.T. De Luca for the preparation of the manuscript and to Mr. A Binda for his cooperation in printing the present Report.

Andres J.P. Klein-Szanto

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A. BIOCHEMISTRY

A.1 Molecular Basis for the Production and Involution of Goiter. I. Action of Iodide on the ^{3}H-leucine and ^{14}C-galactosamine Incorporation into Thyroid Proteins

M.A. Pisarev and L.O. Aiello.

The regulation of protein and nucleic acid biosynthesis is a biochemical event closely related to tissue growth both under physiological and pathological conditions. As far as the thyroid is concerned, regulation is closely related to the development of goiter and tumors. With the purpose of clarifying the stimulatory and inhibitory factors that regulate these processes, an "in vitro" model was developed utilizing labeled compounds to evaluate protein biosynthesis.

Bovine thyroid slices (30-80 mg) were pre-incubated for 30 min in 5 ml of Krebs-Ringer bicarbonate buffer pH 7.6 under 95% O_2 and 5% CO_2 in the presence of the different substances to be tested. At the and of this period a tracer dose (0.5-1 µCi) of ^{3}H-leucine (NEN) for the study of protein synthesis and/or ^{14}C-galactosamine (NEN) for the incorporation of carbohydrate into protein were added. After a pulse of 30-60 min the slices were washed, blotted on filter paper, weighed and homogenized in 1 ml of H_2O. Proteins were precipitated with 0.5 ml of cold 20% trichloroacetic acid. After centrifugation the precipitate was washed twice with 10% TCA digested with protosol (NEN) and counted in omnifluor (NEN). Protein was determined according to Lowry et al. (1). In some studies slices of liver and of submaxillary gland were additionally studied. Soluble protein was separated by centrifuging the homogenate at 105,000 x g for 60 min or at 40,000 x g for 150 min. Thyroglobulin was immunoprecipitated from that fraction according to Vassart et al. (2) with antisera kindly provided by Dr. E. Capalbo (CNEA).

The addition of KI, from 10^{-3} to 10^{-6} M, caused a 50% inhibition in the incorporation of ^{3}H-leucine to total thyroid protein. This effect is organ specificic since it was not observed when liver or submaxillary gland were used. In order to clarify the mechanism of action of the halogen, incubations were performed with 0.5 mM perchlorate or 1 mM MMI (methylmercaptoimidazole). Both drugs blocked the inhibitory action of iodide (Table 1) thus indicating that iodide exerts its action through an organic and intracellular form. The inhibitory potency of KI, and iodothyronines (T_3, T_4) was therefore compared.

It was found that at concentrations of 10^{-6} and 5.10^{-7} M iodothyronines were more potent than KI (Table 2). Moreover KI impaired not only the synthesis of total thyroid protein but that of soluble protein and thyroglobulin as well (Table 3), while it did not alter the incorporation of carbohydrate into the same fraction.

The present results provide new knowledge concerning the mechanism of one of the therapeutic actions of KI and indicate the existence of thyroid autoregulation, probably mediated by the endogenous levels of their hormones. We can therefore conclude that the action of KI on thyroid protein biosynthesis is:

a) specific for the thyroid,
b) exerted intracellularly and by an organic form,
c) probably mediated by thyroid hormones,
d) affects total and soluble thyroid proteins, including thyroglobulin,
e) restricted to protein biosynthesis, without affecting glycosidation.

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TABLE 1
Influence of $KClO_4$ and MMI on the inhibitory action of KI

Experiment	Treatment	TCA precipitable radioactivity DPM/µg protein	p<
A	Control	134.3 ± 27.8	-
	$KClO_4$ 0.5 mM	136.1 ± 17.7	n.s.
	Kl 10^{-4} M	74.5 ± 10.1	0.001
	Kl + $KClO_4$	122.3 ± 4.2	n.s.
B	Control	148.2 ± 37.8	-
	MMI 1 mM	210.4 ± 23.4	n.s.
	Kl 10^{-4} M	78.2 ± 5.2	0.01
	Kl + MMI	127.1 ± 51.0	

Pre-incubation:30 min.[^{3}H]leucine pulse:60 min. Each value represents the average of four slices ± 1 SD.

TABLE 2
Comparative effects of KI, T_3 and T_4 on[^{3}H]leucine incorporation

Experiment	Treatment	TCA precipitable radioactivity DPM/µg protein	p<
A	Control	230.5 ± 46.6	-
	Kl 10^{-4} M	164.2 ± 20.7	0.01
	T_3 10^{-4} M	91.1 ± 5.6	0.001
	T_4 10^{-4} M	143.2 ± 30.7	0.01
B	Control	227.0 ± 28.0	-
	Kl 10^{-6} M	178.3 ± 6.8	0.01
	T_3 10^{-6} M	123.8 ± 5.0	0.001
	T_4 10^{-6} M	114.0 ± 2.0	0.001
C	Control	89.4 ± 8.8	-
	Kl 5.10^{-7} M	113.3 ± 20.5	n.s.
	T_4 5.10^{-7} M	57.5 ± 11.3	0.001

Pre-incubation:30 min. Labelled leucine pulse:60 min. Each value is the average of four slices ± 1 SD.

TABLE 3

Action of Kl on the incorporation of ^{3}H leucine and [^{14}C]galactosamine into soluble proteins

Treatment	Method	[^{3}H]leucine counts/mg protein	p<	[^{14}H]galactosamine counts/mg protein
Control	TCA	209 375 ± 9605	-	15 000 ± 2100
Kl 10^{-5} M	TCA	108 490 ± 1970	0.001	14 900 ± 950
Control	Anti-Tg	31 900 ± 295	-	7575 ± 135
Kl 10^{-5} M	Anti-Tg	23 265 ± 850	0.001	7565 ± 565

Pre-incubation:30 min. Pulse labelling:120 min. For details see Material and Methods. Each value is the average of triplicate or quadruplicate samples ± 1 SD.

A.2 Molecular Basis for the Production and Involution of Goiter

II. Action of KI, Thyronine and Cyclic AMP on the ^{3}H-Uridine and ^{32}P Incorporation into Thyroid RNA

M.A. Pisarev, L.O. Aiello and D.L. Kleiman de Pisarev.

The results of a previous work (1) indicated that iodine exerted its antigoitrogenic action by inhibiting the incorporation of aminoacid into protein. In order to further clarify its mechanism of action the regulation of RNA synthesis was studied utilizing ^{32}P and ^{3}H-uridine as precursors.

Bovine thyroid slices (30-80 mg) were preincubated at 37°C for 30 min. in 5 ml of KBB buffer pH 7.6 under 95% O_2 and 5% CO_2 in the presence of the substances to be studied. The slices were then pulse-labelled with a tracer dose (0.5-1 µCi) of ^{32}P (CNEA) or ^{3}H-uridine (NEN). After 60 min. the slices were washed, blotted on filter paper, weighed and homogenized in 1 ml of H_2O. RNA and oligonucleotides were estimated according to Munro and Fleck (2) in the case of ^{3}H-uridine and following Perry et al. (3) when ^{32}P was used.

To study the half life of RNA the following protocole was used: slices were labelled with ^{3}H-uridine for 10 min. and then extensively washed with KRB. Incubations were followed in the presence of 7 µg/ml of actinomycin D with or without KI at 10^{-5} M. At different times slices were processed for the determination of RNA specific activity.

The radioactivity of the PCA soluble fraction (oligonucleotides) and of RNA was counted in Bray's solution while total RNA was estimated spectrophometrically. Each point was run by quadruplicate and statistical analysis was performed according to Student's "t" test.

Both KI and T_4 at 10^{-5} M decreased the incorporation of ^{32}P and of ^{3}H-uridine into RNA significantly (Fig. 1). No difference in the degradation curve of RNA was observed between control and KI-treated slices. Therefore it was concluded that KI affects RNA synthesis but not its degradation.

With the aim of corroborating whether the mechanism of the action of KI was similar to that observed on protein synthesis the effect of $KClO_4$ and of MMI was studied. Both drugs blocked the inhibitory action of KI (Fig. 2) again indicating that an intracellular and organic form of iodine plays a role.

Cyclic AMP at 2mM caused a significant stimulation of both PCA soluble and RNA radioactivity. This effect was inhibited by 10^{-5} M KI (Fig. 3).

The present results explain the antigoitrogenic action of KI by an inhibition of RNA synthesis. Seed and Goldberg (4) showed that in slices under actinomycin D thyroglobulin (Tg) synthesis proceeds while that of soluble and structural proteins is arrested. In the previous work (1) we found that KI decreases the synthesis of total and soluble thyroid proteins by 50-60% while that of Tg is inhibited by 30%. Our results therefore agree with the above mentioned hypothesis.

The action of KI and of cAMP on the PCA soluble fraction suggest that their inhibitory and stimulatory effects, respectively would be exerted at the precursor nucleotide pool level. A similar action of cAMP was found in bone (5). However our results do not exclude an action at another site. It has been proposed that cyclic nucleotides regulate RNA synthesis by modulating RNA polymerase phosphorylation (6).

In the previous paper (1) it was shown that KI inhibits protein synthesis

through an intracellular and organic form of iodine and that iodothyronines were more potent than KI. The present results confirm that RNA synthesis is also inhibited by an intracellular and organic form of iodine.

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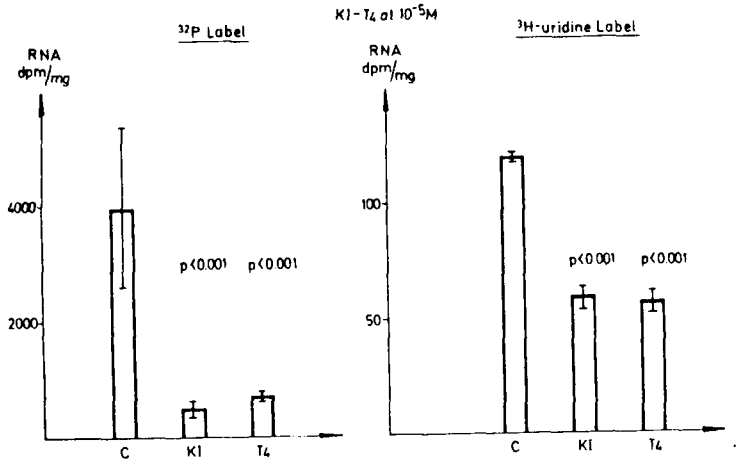

Figure 1

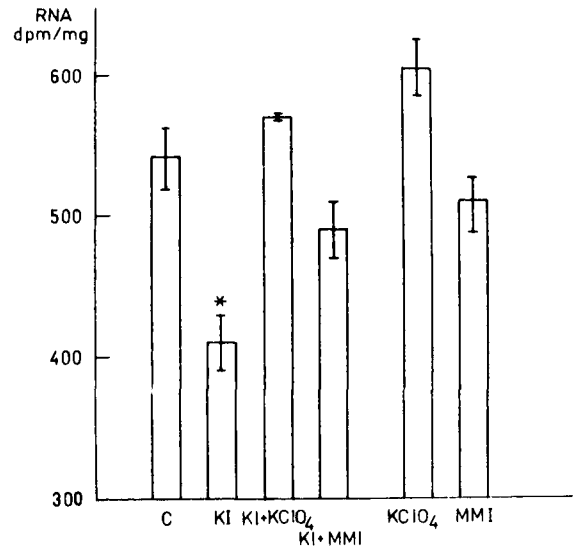

Figure 2

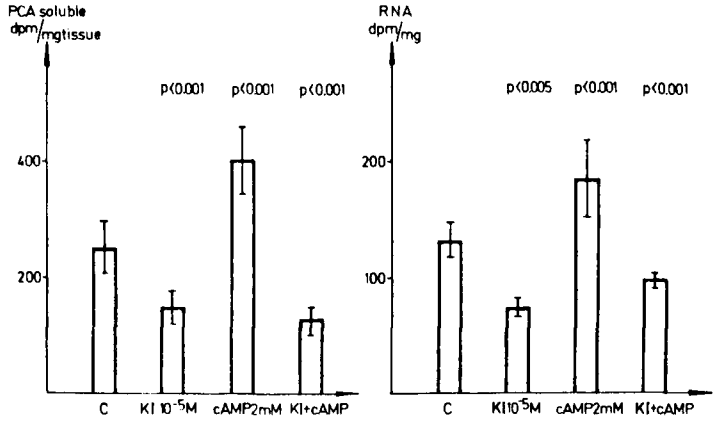

Figure 3

A.3 Molecular Basis for the Production and Involution of Goiter
III. Action of Cyclic Nucleotides on ^{3}H-Uridine Incorporation into RNA and of ^{3}H-Leucine into Protein

M.A. Pisarev & D.L. Kleiman de Pisarev.

TSH regulates thyroid function via cyclic AMP (1). This nucleotide stimulates the incorporation of aminoacids into protein, in vivo (2) and in vitro (3, 4). Another nucleotide, cyclic GMP, also stimulates this parameter and guanylate cyclase and cyclic GMP dependent protein kinases have been found in the thyroid. TSH stimulates RNA synthesis and this effect is reproduced by cAMP (4). In the present study we analyze the action of cGMP and cCMP on thyroid protein and RNA synthesis.

Beef slices were incubated inKRB. As already described the solution contained 1 mM caffeine and,when protein synthesis was studied, was supplemented with 2 mM L-leucine. Test substances were included during the 30 min. preincubation. Slices were pulse labelled for 60 min. with either ^{3}H-leucine (proteins) or ^{3}H-uridine (RNA).

Protein synthesis:
TCA precipitable radioactivity was counted in omnifluor and protein estimated according to Lowry (5).

RNA synthesis:
was measured according to Munro & Fleck (6) and radioactivity counted in Bray's solution.

Statistical analysis was performed following Student's "t" test.

Both cAMP and cGMP stimulated the incorporation of ^{3}H-uridine into RNA while 5' AMP and 5' GMP were ineffective. This action of cyclic nucleotides was inhibited by actinomycin D (4 µg/ml). (Fig. 1).

Cyclic CMP in doses from 0.35 to 1.5 mM progressively decreased both RNA and oligonucleotides radioactivity (Fig. 2).

When the interaction between cAMP and cCMP was studied it was found that cCMP blocked both protein and RNA synthesis stimulated by cAMP (Fig. 3).

The present results show that both cAMP and cGMP mimic the action of TSH on RNA synthesis. Although both nucleotides have parallel effects on thyroid function their intracellular concentration is regulated by separated mechanisms. The inhibition caused by actinomycin D suggest that they act at a pre o at translational level. However the effect on the PCA soluble fraction would indicate a stimulation at the oligonucleotide level (4) while another possibility would be the RNA polymerase phosphorylation.

Cyclic CMP progressively decreased synthesis and blocked cAMP stimulation. Very little is known about this nucleotide. It was been isolated from leukaemia L-1210 cells and it could play a role in tumor growth, opposite to that of cAMP. Until now only positive modulators of thyroid function were known (cAMP and cGMP). The results herein presented provide the first indication of the possible existence of negative modulators of the thyroid and propose the hypothesis of the role of cCMP in this sense.

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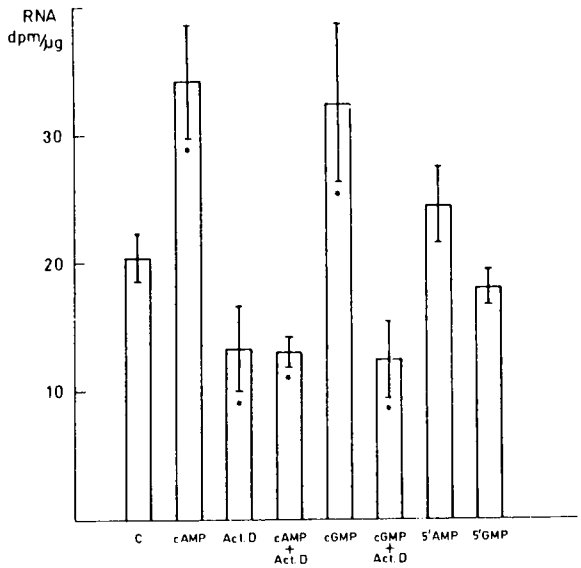

Figure 1

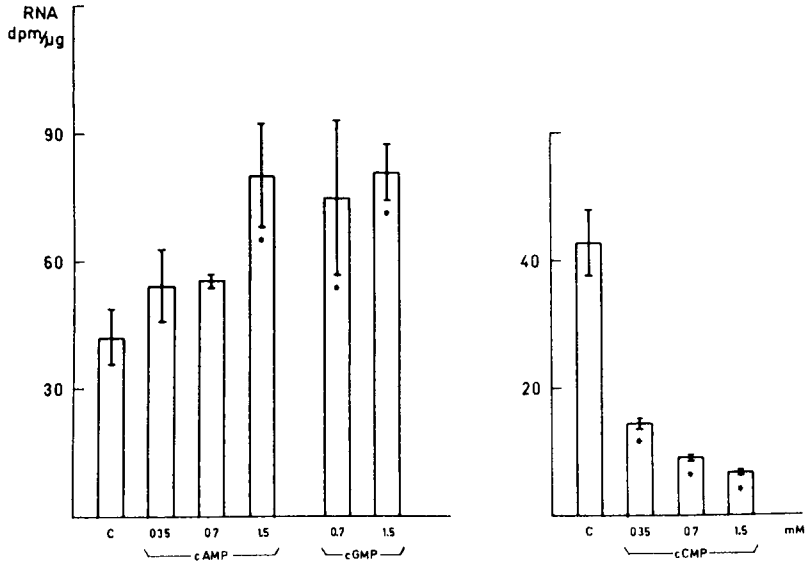

Figure 2

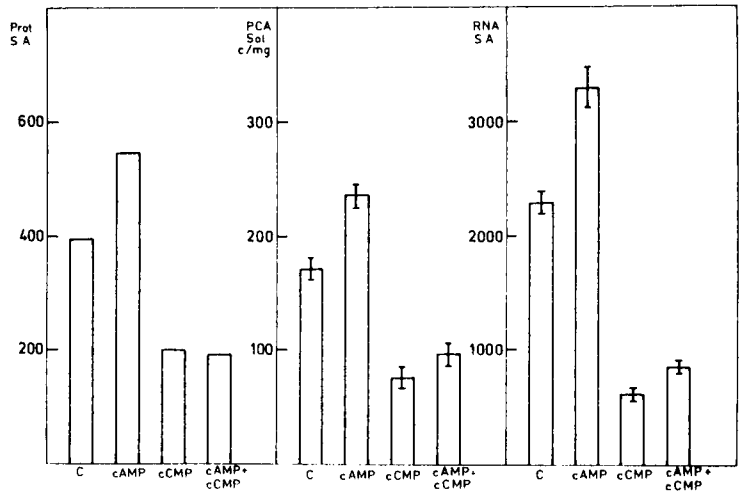

Figure 3

A. 4 Metabolism of ^{131}I in Studies of Antithyroid Drugs

E.P.K. de Valmaggia, R. Valsecchi, R.P. Gagliardi, M. de. C. A. de Pirovano,* M. Pisarev and N. Altschuler.

The treatment of hyperthyroidism during pregnancy is still a matter of controversy. The present studies were performed with the aim of evaluating the different drugs used in therapeutics, especially in relation to the probable damage to the fetus.

Wistar female pregnant rats, 170 ± 10 gr body weight, were distributed in three groups:

a) controls
b) propylthyouracil)PTU) 3 mg/day s.c. from the onset of pregnancy
c) methylmercaptoimidazol (MMI) 0.3 mg/day s.c. from the onset of pregnancy.

The administration of these drugs was continued during lactation and thyroid function was studied in the offspring at 28-36 days of age. The animals received, 24 h. before sacrifice, a tracer dose of ^{131}I.

Body weight, thyroid weight, ^{131}I uptake, circulating radioactivity, PB ^{131}I and the thyroid: serum (T/S) ratio were determined by conventional methods.

Thyroids were homogeneized with a Potter-Elvehjem apparatus in 1 ml of TRIS-HCl buffer 0.1 M pH 8.5 and digested for 48 h. with 1% pancreatine (Difco). Aliquots were spotted on Whatman 1 paper and developed on n-butanol; ethanol: 1N ammonium hydroxide (5:1:2; BEA) for 22 h. The distribution of radioactivity in the chromotogram was determined. Neurologic maturation was evaluated by the swimming test (Schapiro) (Fig. 1).

A notable difference was observed in the evolution of body weight of the rats whose mother had been trated with PTU, while no alteration was found in the MMI-group. Thyroid weight was significantly increased, as well as thyroid ^{131}I uptake, in the PTU-rats. When the values of uptake were normalized for thyroid and body weight, they were significantly decreased in the PTU-group. Circulating radioactivity and PB^{131}I were increased and chromatograms showed a decrease in the percentage iodothyronines in the PTU-rats.

In order to better simulate an experimental hyperthyroidism another group of animals was treated simultaneously with the antithyroid drugs plus 5 µg/day of l-thyroxine (T_4).

The administration of l-T_4 partially reverted the effect of the PTU, shifting body weight and the other parameters towards normal.

It is known that PTU acts peripherally by increasing binding of thyroid hormones to carrier proteins and by decreasing their utilization by tissues.

It is likely that under our experimental conditions hypothyroidism developes both with a decrease of iodothyronines synthesis, as shown in the chromatograms, as well as by inhibition of peripheral action.

With the aim of learning whether the transport of PTU from mother to conceptors is performed via placenta or by the milk, PTU was administered to the mother either during pregnancy till delivery or from the first day of birth.

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* Jardín Zoológico de la Ciudad de Buenos Aires.

The results obtained are shown in Table II. The difference observed between both experimental groups and the controls was remarkable. A significant increase of thyroid weight was found in the animals treated during lactation as compared to those treated during pregnancy. Thyroid ^{131}I uptake was greatly decreased in the same group. The effect of PTU on thyroid parameters is more apparent in the animals treated during lactation. However when the swimming test was performed a delay was found in neuromuscular maturation of the offspring of animals treated during pregnancy, which would suggest a greater sensibility of SNC during this period.

The birth of children with goiter and hypothyroidism has been reported from mothers treated with antithyroid drugs. The present results suggest the convenience of the use of MMI since no significant alterations were found in this group. However since these studies were performed in the rat, more studies, including those of humans, will be necessary before arriving at a definitive conclusion.

TABLE I

	Thyroid weight mg/100 g BW	Thyroid uptake % dose	Thyroid uptake $\frac{\%\ dose}{mg/100\ mg\ BW}$	Plasma radioactivity % dose/ml	T/S	$PB^{131}I$ % dose/ml
Normal	8.28 ± 1.34	1.96 ± 0.18	0.27 ± 0.06	0.38 ± 0.06	0.79 ± 0.15	0.05
MMI	8.69 ± 1.36	1.44 ± 0.44	0.17 ± 0.05	0.66 ± 0.15	0.50 ± 0.17	0.06
PTU	30.36 ± 7.25	2.66 ± 0.30	0.09 ± 0.02	1.41 ± 0.26	0.25 ± 0.08	0.140 0.170

TABLE II

	Thyroid weight mg/100 g BW	Thyroid uptake % dose	Plasma radioactivity % dose/ml	T/S	$PB^{131}I$ % dose/ml
Normal	9.8 ± 3.4	1.45 ± 0.21	0.32	0.75 ± 0.15	0.065 ± 0.010
MMI + T_4	10.5 ± 0.2	1.38 ± 0.15	0.38	0.82 ± 0.16	0.068 ± 0.022
PTU + T_4	33.5 ± 3.6	2.14 ± 0.72	0.64	0.38 ± 0.09	0.140 ± 0.050
T_4	9.4 ± 2.6	0.51 ± 0.30	0.39	0.24 ± 0.04	0.066 ± 0.034

TABLE III

	Body weight gr	Thyroid weight mg/100 g BW	Thyroid uptake % dose/100 g BW	Plasma Radioactivity % dose/ml	T/S	$PB^{131}I$ % dose/ml
C	59.4 ± 5	7.68 ± 0.80	0.47 ± 0.21	0.47 ± 0.15	0.64 ± 0.05	0.13 ± 0.03
PTU (Lactancia)	30.4 ± 7	26.91 ± 3.8	0.09 ± 0.03	2.43 ± 0.49	0.04 ± 0.02	0.50 ± 0.08
PTU (Gestación)	30.3 ± 12	13.17 ± 1.84	0.27 ± 0.05	2.11 ± 0,21	0,13 ± 0,03	0.52 ± 0,05

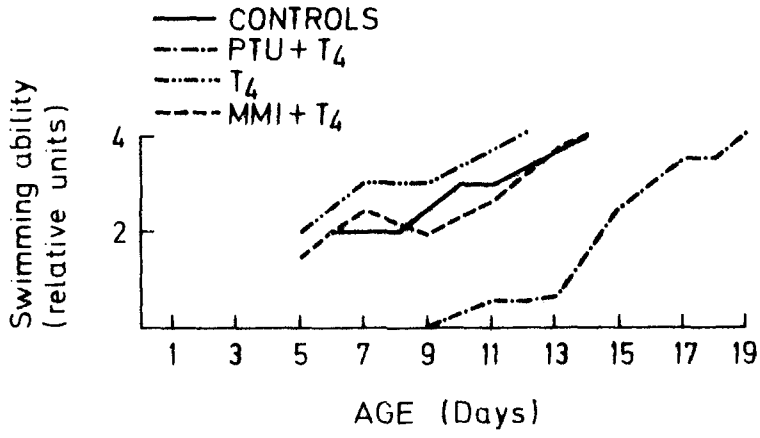
CONTROLS
PTU + T4
T4
MMI + T4
Swimming ability
(relative units)
4
2
1
3
5
7
9
11
13
15
17
19
AGE (Days)

Figure 1

A. 5 Correlation Between the Estimated Molecular Weight and the Immunological Properties of ^{125}I-TSH

M. Barmasch,* S.E. Quiroga,** V.A. Ciscato,** H. Kurcbart,* S.V. de Giacomini,** N. Altschuler* and R.A. Caro**

In order to continue our studies on the methodological standardization of radioimmunoassay procedures, in the present study we try to establish a relationship between the physicochemical properties of each fraction and its immunological behaviour. Thus, we studied, for the case of human TSH, the correlation between the estimated molecular weight of each fraction and its own specific binding by means of the Dextran-coated charcoal ratio (DCR), its specific binding to the antiserum at a dilution which corresponds to a fifty per cent binding, as well as its displacement curve. Thyrotropin was labelled with ^{125}I-sodium iodide (NEZ-033H, New England Nuclear Corporation), following the method proposed by Caro et al. (1).

A Sephadex G-75 (Pharmacia, Uppsala) column calibrated with four molecular weight standards was used to determine the MW of the labeled hormone. The first tubes with measurable radioactivity correspond to products having an estimated MW of 70,000 dalton or more, indicating a probable polymerization of the hormone (Sherman et al.; Kieffer et al.). The most important radioactive peak was observed between 30,000 and 42,000 daltons (Fig. 1). Since the native TSH has a MW of 28,000 (4) it should be found at the posterior shoulder of the main peak, and also labeled polypeptides are obtained, with a MW down to 15,000 or less (Fig. 1). Similar elution patterns are obtained with labeled TSH of different species (Table I).

The non-specific binding of each fraction was determined in the abscence of antiserum, the so-called dextran-charcoal ratio, DCR

$$DCR = \frac{R_S}{R_p} \quad \begin{matrix} \text{Radioactivity in supernatant} \\ \text{Radioactivity in precipitate} \end{matrix}$$

As it can be observed in Fig. 2, DCR has a value of around 0.25 for MW of 27,000 with values greater than 1 at high molecular weights, whereas for those fractions with small MW, down to 15,000, the DCR is low.

In order to fit these results into a quantitative scheme specific/non-specific ratio (SNR), defined as the ratio between the specific binding in the abscence of standard hormone and DCR

$SNR = \frac{(B/F)_o}{DCR}$ is plotted in function of MW of each fraction (Fig. 2). A single peak at a MW similar to that of the native hormone can be observed.

These results are confirmed by the logit plots using labeled hormones of

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different molecular weights (Figure 3). The logit plot with a ^{125}I-TSH having a molecular weight of 28,000 daltons, shows a well defined straight line with the correct slope. With the same fraction we determined, by a Scatchard plot (Figure 4), the mean affinity constant, which gave a value of 1×10^9 l/mol.

Several authors utilize the dextran coated charcoal technique of the binding to an excess of antiserum in order to test the quality of the labeled hormone. On the other hand, others work with a pool of the different protein fraction eluted from the column, without taking into consideration that the Sephadex gel separates fractions of different molecular weights (5). These fractions show a heterogeneous physicochemical behaviour as well as dissimilar antigenic properties as demonstrated by our results.

In the present work we demonstrate that the clearest way to analyze this conditions consists in the determination of the ratio between the corrected specific binding and the non-specific adsorption to the dextran coated charcoal (SNR). As is to be expected, the highest SNR value, i.e., the best one, was found to be coincident with the fraction having an estimated molecular weight similar to that of the native hormone. Our results also show that a good correlation exists between the DCR and the estimated molecular weight of the eluted fraction. DCR values ranging between 0.2 and 0.3 were found for the fraction having the best immunological properties (molecular weight 28,000).

When the binding to an excess of antibody is used as a quality control, the obtained results may be inadequate, since the equilibrium of the antigen-antibody reaction may be shifted due to the excess of antibody. This implies that if the complex is formed at all, its quantity is smaller than the theoretical one. Thus, erroneous conclusions may be derived concerning the quality of the labeled hormone.

From the practical point of view we may conclude that, if the conditions of the column chromatography are maintained constant to each fraction corresponds a given molecular weight, DCR and SNR. Our results demonstrate that the fast and simple determination of the DCR can be applied to choose the optimal fraction to be used in the radioimmunoassay.

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2.- Sherman, L.A., Harwig, S. & Hayne, O.A., Int. J. Appl. Radiat. Isotopes, 25:81, 1974.

3.- Kieffer, J.D., Weintraub, B.D., Leeman, S. & Maloof, F., Acta Endoc., 76:495, 1974.

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MW estimation of TSH from different species			
HUMAN	RAT	OVINE	BOVINE
70.000	70.000	70.000	70.000
60.000	54.000	42.000	54.000
33.000	34.500	18.000	32.000
15.000	20.500	4.000	14.000
8.000	14.000		4.000
4.000	10.000		
	4.000		

Table I

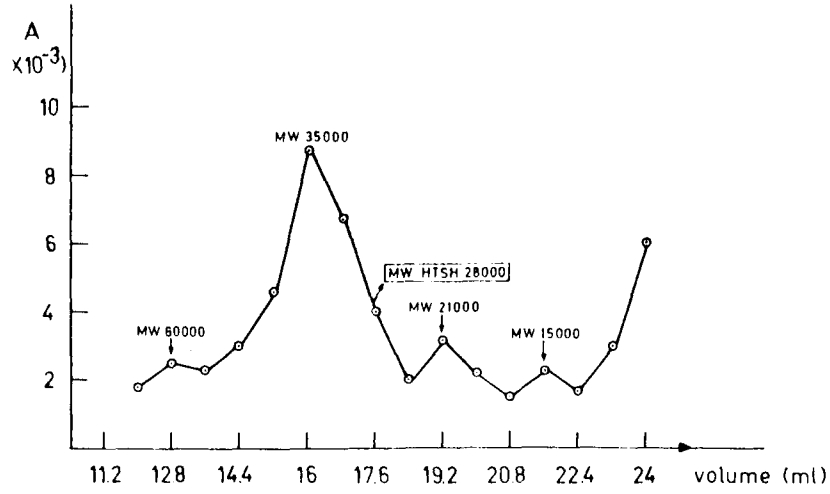

Figure 1

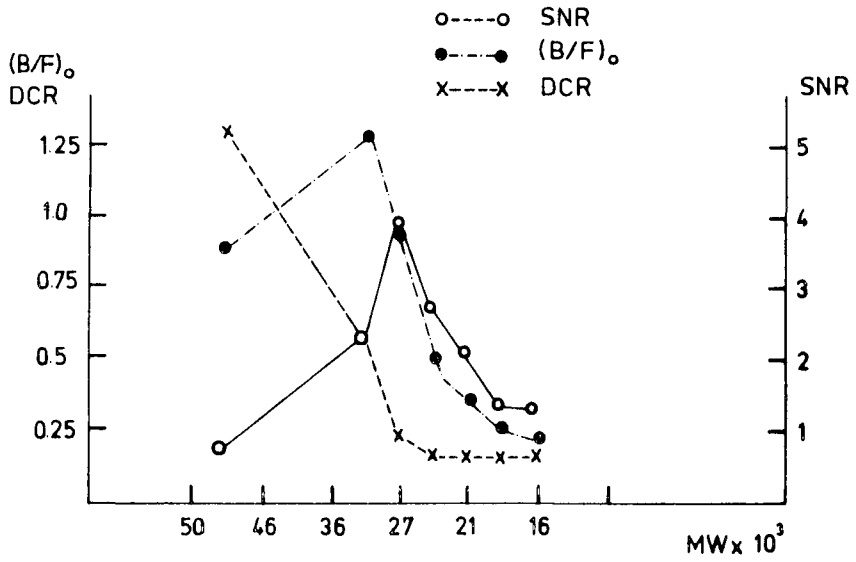

Figure 2

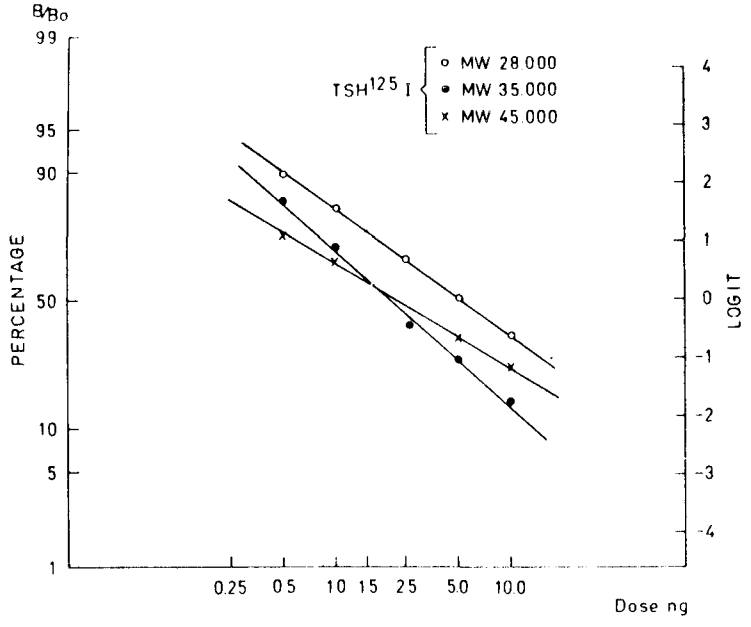

Figure 3

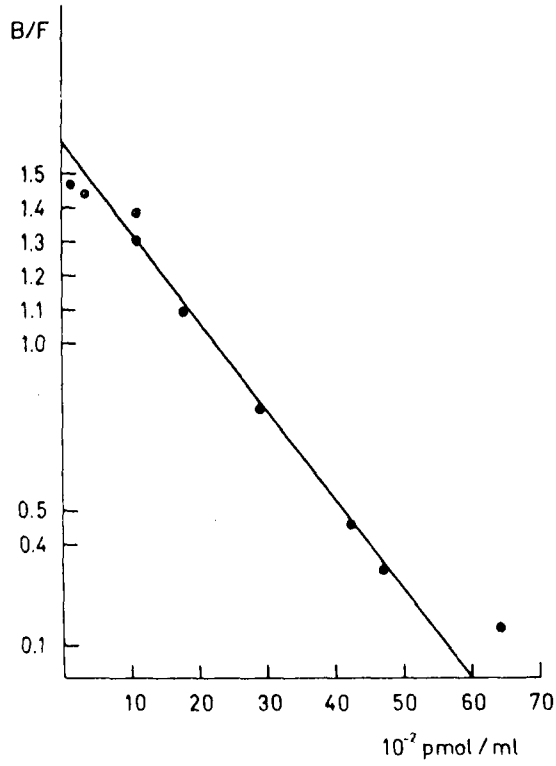

Figure 4

A. 6 Biological Studies Using Radioactive Tracers in an Argentine Primate

R.M. Valsecchi, G. Sala,* V. Dahl,* C. Bergada,* O.J. Degrossi,**
C.P. Lantos** and N. Altschuler.

A new-world primate, Alouatta caraya, has been employed as a biological model for the study of various thyroidal and adrenal parameters; this species has been chosen in view of its phylogenetic vicinity to man.

Stable iodine in the thyroidal tissue amounted to 0.95 and 1.4 µg/mg Kinetic studies with ^{131}I showed a high turnover rate as compared to man. Initial values (10-60 min) were equal to 0.3% dosis/ml plasma and decreased to 0.006-0.0006% dosis/ml after 24 hours. Thyroid uptake was high after one hour (20-40%) but decreased sharply after a period of 24 hours (4-15% dose).

Neither stimulation (TSH) nor discharge (ClO_4K) show any effect on ^{131}I uptake nor on kinetic studies, intra-thyroidal distribution of ^{131}I yielded values from 0.4 to 1.9% in the lysosomal fraction. The supernatant submitted to a linear sucrose gradient, showed a radioactivity peak at the fraction of 19S, coincident with thyroglobulin.

Plasma-half life of $T_4{}^{125}I$ thyroxine compartment and daily metabolic rate values were all lower than in humans. However, when the latter were expressed as µg/kg body weight/day, these values were coincident with those found in men. Kinetic studies with $T_3{}^{125}I$ carried out in a young primate showed a half-life similar to that of man; but compartmental and metabolic rate values were lower; when the latter was expressed as a function of body weight, the values were similar. Carrier studies pointed to the absence of a specific binding protein for thyroidal hormones.

Adrenal steroidogenesis was investigated in 2 females ans 1 adult male of this species, which is known not to reproduce in captivity. The studies were partially aimed at investigating one possible reason for this lack of reproduction.

Adrenal slices of approximately 100 mg were incubated for 30, 120 and 240 minutes in Krebs-Ringer-bicarbonate-glucose buffer at 37°C, pH 7.2. Precursors were pregnenolone 7-3H (PR) (sp.act.62.5 mCi/mg) (I), or 7-3h-PR+4-14C progesterone (P), both having a specific activity of 0.167 mCi/mg (II). In case I the following radiometabolites were quantitated by a derivative-dilution method: 17 α hydroxyprogesterone (17 α OHP) deoxicorticosterone (DOC), corticosterone (B), cortisol (F), 11-deoxicortisol (S), cortisone (E), 18 hydroxycorticosterone (18OHB), aldosterone (ALDO) and two unknown compounds, X_1 and X_2, with an RB in toluene-propylene glycol of 1.4 ± 0.1 (N=6), correponding to 5-dihydrocorticosterone. Endogenous cortisol (F) was determined by protein competition and corticostorene (B) by fluorometry. In case II the same compounds were characterized and metabolic pathaways were investigated. It could be shown that 17 α OHP, S, F and E proceed mainly from PR and that DOC, B, 18OHB and X_2 proceed mainly from P.

At 240 minutes percentages from PR for duplicates were the following: 17 α OHP:71±2.-S:58±1.-F:56±2.-E:55±1.-DOC:10±1.-B:12±1.-18OHB±16±1.-X_2:14.

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Compound X_1 proceeds 71±1% from pregnenolone. F/X_1 ratios in (I) are 4.1 and 6.3.X_1 on thin-layer plates (ethyl-acetate:hexane 3:1) has an Rf of 0.77±0.01 (N=13). In this system the Rf of B is 0.27 and that of X_2, 0.38±0.01 (N=6). In methylene choride;methanol(159:2) the Rf of dihydrocorticosterone is 0.43, and that os X_1 is 0.35. The compound can be acetylated and the acetate, in this last system, has an Rf of 0.80. The identity and metabolic situation of this compound is unknown.

	$[T_4]$ µg/100ml	$T_{1/2}$ día	E.D. %p.c.	Q_{T_4} µg	T.D.D µg/día	T.D.D µg/kg/día
Hombre	8,7	7-9	15,2	859,6	89,2	1,4
Alouatta carayá	5,3	1,9 joven	12,1	18,8	7,4	2,2
	4,9 (6)	2,9 adulto	14,4	62,6	14,9	2,1

	$[T_3]$ ng/100ml	$T_{1/2}$ día	E.D. %p.c.	Q_{T_3} µg	T.D.D. µg/día	T.D.D. µg/kg/día
Hombre	100-200	1,2	65	63	37	0,57
Alouatta carayá	180	1,3	44	2,6	1,4	0,43
	86 (7)					

Table I

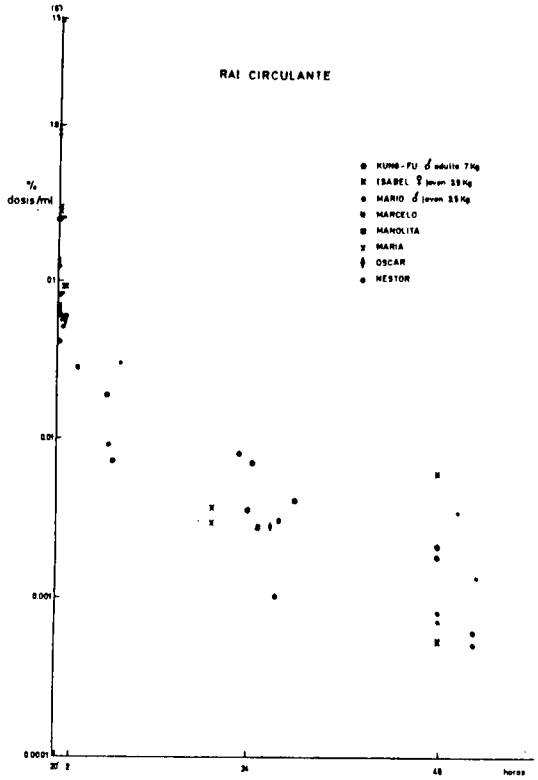

Figure 1

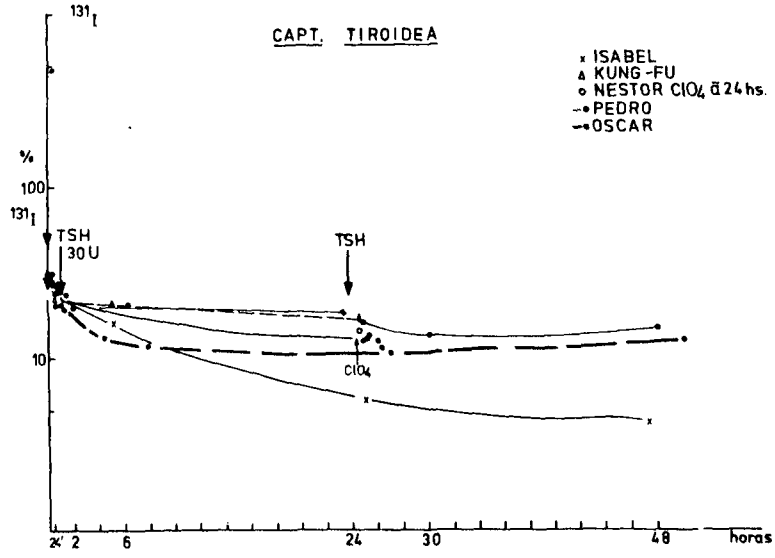
131I
CAPT. TIROIDEA
x ISABEL
Δ KUNG-FU
o NESTOR ClO4 á 24 hs.
-•PEDRO
-•OSCAR
%
100
131I
TSH
30 U
TSH
10
ClO4
2' 2
6
24
30
48
horas

Figure 2

A. 7 Studies with Copper-67 and Copper-64 in Conditioned Copper-Deficient Ruminants. Preliminary Results

N.A. Marcilese,* H.D. Figueiras* and R.M. Valsecchi.

Copper deficiency in ruminants under grazing conditions, caused by an excessive intake of molybdenum and inorganic sulphate, is a most important trace mineral deficiency in large areas of the world. In previous work with sheep we studied some of the mechanisms by which copper metabolism is affected under such dietary conditions together with the sites affected. It was demonstrated that the ability of the liver to store ^{64}Cu and the utilization of the tracer in ceruloplasmin production was impaired. A failure in the synthesis of cuproproteins by the liver was postulated to explain the dysfunction. In addition, it was shown that urinary losses of copper were significantly increased, explaining in part at least why body depletion of copper occurs in deficient animals. It was also suggested that the faecal endogenous excretion of copper was higher in the molybdenum sulphate fed animals, although this fact could not be clearly demonstrated because of the fast decay of the tracer. An important limiting factor in the above-mentioned studies, was the short half-life of ^{64}Cu, its period 12.8 hours makes this isotope useful for short-term studies only. The avalability of ^{67}Cu, of 61.8 hours period, in combination with ^{64}Cu, allowed new studies to be undertaken to gain further knowledge of the interaction of molybdenum-sulphate upon copper metabolism in ruminants. The parametes studied were: the plasma clearance of ^{67}Cu (cattle and sheep); the apparent gastrointestinal absorption and the faecal endogenous excretion of ^{67}Cu (sheep); the plasma clearance of ceruloplasmin-labelled-^{64}Cu in vivo (cattle); and the intracellular distribution of ^{64}Cu in the liver of control cattle and sheep and deficient cattle.

In male young cattle (Hereford) and wethers (Corriedale), copper deficiency was induced by feeding the animals with a diet of ground first quality alfalfa hay, to which sodium molybdate and sodium sulphate were added to obtain a level of 50 ppm of molybdenum and 0.4% of sulphate respectively (cattle), or 75 ppm of molybdenum and a similar level of sulphate for the sheep diet, on a dried matter basis. Levels of copper in the alfalfa hay ranged from 8 to 14 ppm. Control animals were fed with the same diet without supplemental molybdenum and sulphate. The feeding period required to induce a severe copper deficiency varied from six to nine months. The feeding of the diet was mantained during the studies. Apparent absorption of ^{67}Cu from the gut was determined in two control wethers kept in metabolic cages, after orally dosing the animals with 100 µCi of the tracer (copper chloride, carrier free). The whole excreta was then collected for 12 days by means of bags. Plasma clearance of ^{67}Cu and its faecal endogenous excretion were studied after injecting intravenously 200 µCi to one control and to one deficient sheep. Samples were collected daily during a similar period. Plasma clearance of ^{67}Cu was also determined in one control and one deficient steer during 12 days, after the intravenous administration of 600 µCi. The half-time of ceruloplasmin-^{64}Cu in plasma was determined in two control steers, after the injection of 600 ml of plasma obtained from normal animals that had been injected the previous day with 20 µCi of ^{64}Cu (copper chloride, specific activity 10 mCi per mg). This procedure has been described elsewhere. The intracellular distribution of ^{64}Cu in the liver was carried out in duplicate samples obtained 24 hours after injecting 20 mCi of the tracer into one control and into one deficient steer and 5 mCi into a normal sheep. The animals were slaughtered, the liver quickly removed, and 8 g of tissue were cut into small pieces and homogenized in a Potter-Evehjem

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* Div. Agrop. C.N.E.A.

homogenizer with a Teflon pestle using a 0.25 M sucrose solution. The final concentration of the homogenate was 4 ml of sucrose solution per gram of tissue. The homogenate was centrifuged at 800 x g for 10 min, the supernatant was centrifuged at 14,000 x g for 30 min and the second supernatant was centrifuged at 20,000 x g for 30 min. The last supernatant was then centrifuged at 105,000 x g for one hour in a LS-65 model Beckman ultra-centrifuge. The fractions thus obtained were: nuclei and cell debris, mitochondria, lysosomes, microsomes and soluble fraction respectively.

The apparent gastro-intestinal absorption of ^{67}Cu in sheep was similar in both groups of animals: 7.9 and 6.7% of the administered dose for deficient and control wethers respectively. On the other hand, faecal endogenous excretion of the tracer injected intravenously was almost twice as high in the deficient animal as in the control: 6.2 and 3.5% respectively.

Plasma clearance of ^{67}Cu showed striking differences between copper-deficient and control animals, as well as between species. Sheep presented a clearance curve that could be fitted by a threeterm exponential function, identical to those previously verified by means of ^{64}Cu during a 30 hour period. In both studies the deficient animals showed removal of the tracer from the plasma at a very low rate when compared to the controls. In cattle the same was true, although the difference between the rates of plasma clearance in deficient and control steers was even greater.

The results presented here clearly indicate that in cattle the negative effect of dietary molybdenum and sulphate upon this parameter of copper metabolism is much more pronounced than in sheep.

The longer observation period of the studies with ^{67}Cu also allowed one to verify some particular difference in the rate of ceruloplasmin production between normal cattle and sheep. In this last species a relatively small amount of the activity cleared from the plasma about 2.3%, was returned to the circulation and reached a peak about 24 hours after the injection of the tracer. At this time the radioactivity present in the ceruloplasmin fraction of the plasma copper was around 66% of the total. A simple exponential function fitted the removal of such radioactivity from the plasma, with a half-time of about 70 days. This fact suggest a low but continuous incorporation of the tracer into ceruloplasmin form and its sunsequent release to the circulation.

Repetitions with ^{64}Cu showed that in some cases values up to 20% of the radioactivity injected return to the circulation only 24 hours after the administration of the isotope. At this time more than 85% of such radioactivity is present in the ceruloplasmin fraction of the circulating copper. The biological significance of the differences revealed in the rate of ceruloplasmin production and its removal from the blood between cattle and sheep must be further investigated. However, it can be tentatively postulated that such differences could explain the higher susceptibility of cattle to dietary molybdenum and sulphate.

The cubcellular distribution of ^{64}Cu in the liver samples of control and deficient steers shows some differences among several fractions, as can be seen in Table I, although greater differences can be observed between normal cattle and sheep.

However, the lack of other data for normal animals, as well as limited knowledge about the metabolism of copper inside the liver cells, mainly in relation to the role that each fraction plays in the transport of copper and the synthesis of cuproproteins, make difficult even a tentative interpretation of such preliminary data. These results suggest that further research on this subject could be worth while, as previous research and the results presented here strongly indicate that the liver plays a key role in the metabolism of copper in the animal body.

TABLE I

%	Control cattle	Defficient cattle	Control sheep
Nuclei and debris	17,30	26.41	50.00
Mitochondria	13,91	15.48	29.10
Lysosomes	2,10	1.70	2.80
Microsomes	1,15	2.10	4.20
Soluble fraction	65,47	54,40	12.90

A. 8 Radioimmunoassay of Digitalics. Its Normalization

M. Barmasch, L.N. Pérez and N. Altschuler.

The fundamental aim of this work was to study the methodology of radioimmunoassay of digitalics in different biological media, as well as the behaviour of the following compounds: digoxin, (D) β-methyldigoxin, (β-MD) against the same antibody.

The methodology thus developed was applied to pharmacological studies in order to evaluate the passage of β-MD, D and digitoxin (DT) through the blood-brain barrier.

Previous to the determination of these compounds in cerebro-spinal fluid (CSF) and sera, studies were made in order to standardize the method. Different commercial Kits of Digoxin were tested and standard curves were run with human serum and the lyophilized sera supplied with some Kits. Logit-log plots showed different behaviour of the lyophilized and the fresh sera, giving non-superposable curves. This difference is possibly due to probable denaturation of the serum proteins during the lyophilization process. It is important to remark the advice of the commercial Kit suppliers to use fresh sera to run the standard curves in order to obtain the same behaviour as the test serum.

Protein influence was studied by running dose-response curves with serum, plasma and CSF, showing curves by logit-log plot, with different slopes (Fig. 1). From this and the above mentioned data we conclude that we should run the standard curves in the same biological medium under the same conditions as the sample under testing. This fact is of real significance when carbon-dextran is used as adsorbant in the separation of free from bound digitalics. These results confirm the work of Albano et al. (1) as well as those from our laboratory done in sera of different species (2).

When recovery of β-MD was studied, either in serum or in CSF, we got, values higher than the expected. Therefore, dose-response curves were run, utilizing β-MD as standard to compare with the D standard supplied in the Kit, showing a higher binding value for β-MD compared to D. Therefore, dose-response curves were run and, when analyzed in logit-log system, different slopes were obtained with antisera of the Kits utilized (Fig. 2).

In order to attemp an explanation of this behaviour, we applied the Scatchard analysis (3) to these dose-response curves, showing that the antibody can discern between the β-MD and the D molecules. It can be observed that, when antibodies of Kits of different commercial sources are used, they present some abnormalities in the Scatchard plot. Figure 3a is the Scatchard plot obtained with a Kit of Digoxin ^{125}-I in CSF and we can observe a homogeneous behaviour of the antibody with the digoxin standard. With the β-methyldigoxin standard, on the other hand, we observe more than one slope, showing more than one immunochemical entity. Figure 3b shows that the behaviour of the other antibody of the ^{3}H-digoxin Kit is not homogeneous. In the β-methyldigoxin plot, although a K_1 affinity constant predominates, we can suggest the presence of other components with different constants, and with the digoxin standard we can also see a heterogeneous behaviour. These data would indicate that each antibody is able to recognize, or not, the different metabolites of these cardiotonics. In case of an immuno-recognition each antibody will react with a given affinity contant.

These results indicate that in biological samples from patients we will get different values if we interpolate in the curve obtained using β-methyldigoxin as a standard.

Furthermore, studies were done to establish the presence of these digitalics in CSF. Table I shows the results obtained with the patients treated with β-MD. The levels of this compound in CSF were non-detectable in 4 patients and in the others ranged betwwen 1.3 and 2.7 ng/ml, when the radioimmunoassay was done with β-MD as a standard. When Digoxin, supplied with the Kit, was used as a standard, the values were :non-detectable" in three patients and from 0.25 to 1.25 ng/ml in the other. The ratio of the values when both compounds, β-MD and Digoxin, were used as standars ranged between 2.2 and 3.7 in the different patients studied. This variation can be ascribed to the presence of several metabolic compounds, which would react with different affinity constants against the same antibody, since a different binding of these metabolites can be expected as suggested by the Scatchard analysis.

In three patients with "non-detectable" values for β-MD in CSF, determinations of this compound in serum were done and the values obtained were: 0.3, 0.66 and 3.5 ng/ml. On the other hand, when digoxin was used as a standard, the serum values were "non-detectable", 1.8 and 1.6 ng/ml, respectively.

It must be emphasized that of 10 patients digitalized with digitoxin none of them showed that this compound crosses the blood-brain barrier. The circulating levels varied between 5.6 and 23.0 ng/ml (Table II).

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2.- Barmasch, M., Cembal, S., Kurcbart, H., Ciscato, V., Veira, S., Quiroga, S., Altschuler, N. and Caro, R., Acta Physiol. Latinameric., 23:3, 1973, Abstr. 290.

3.- Scatchard, G., Ann. N.Y. Acd. Sci., 51:660, 1949.

TABLE I

β-methyldigoxin treatment

Patient	Cerebro spinal fluid			Serum		
	Digoxin standard	β-methyldigoxin standard	Ratio	Digoxin standard	β-methyldigoxin standard	Ratio
Cac.	N.D.*	N.D.	-	1.6	3.5	2.19
E	0.25	N.D.	-	N.D.	0.3	-
Mo.	N.D.	N.D.	-	1.8	0.66	0.36
N	1.25	2.7	2.2			
Mu.	0.68	2.0	2.9			
D	0.5	1.3	2.6			
R	0.6	1.5	2.5			
Can.	0.4	1.5	3.7			
F	N.D.	N.D.				

* N.D.: Non detectable.
Values expressed as ng/ml.

TABLE II

Digitoxin treatment

Patient	Digitoxin (Serum)	Digitoxin (C.S.F.)
M	23.0	N.D.*
S	15.0	N.D.
G	9.5	N.D.
Ro	18.0	N.D.
B	17.0	N.D.
L	10.0	N.D.
AR	5.6	N.D.
P	8.0	N.D.
CR	19.0	N.D.
NR	16.0	N.D.

* N.D.: Non detectable
Values expressed as ng/ml.

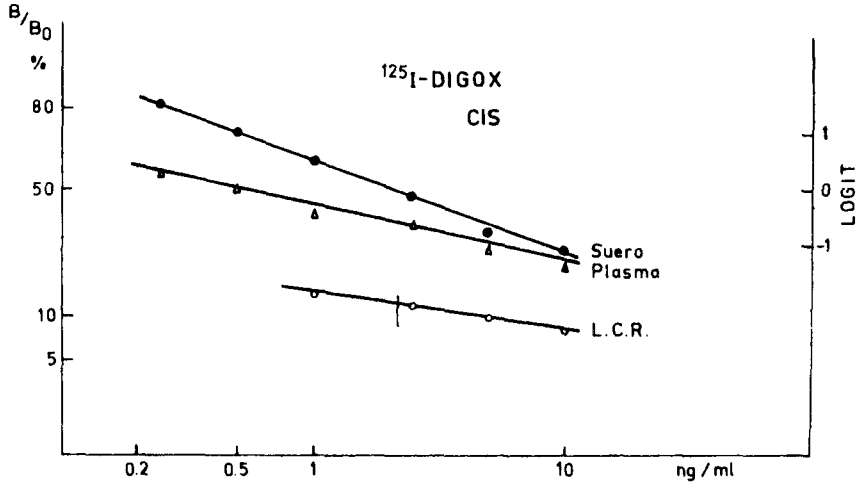


Figure 1

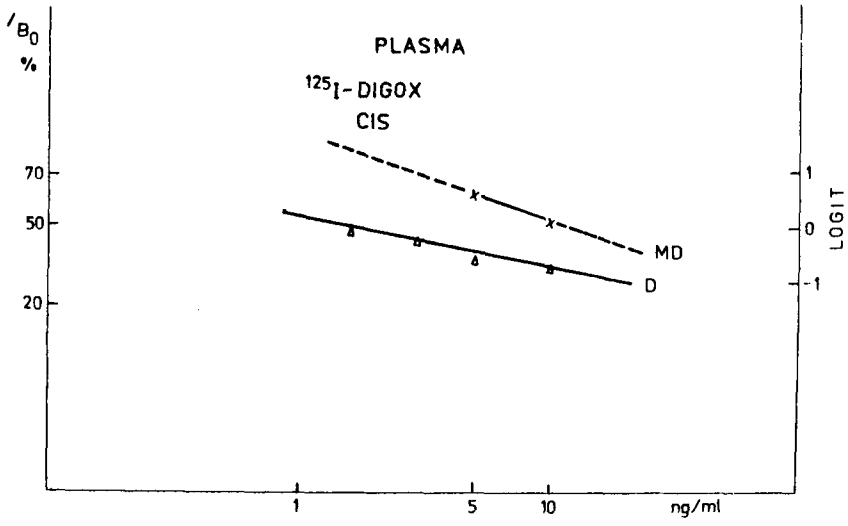
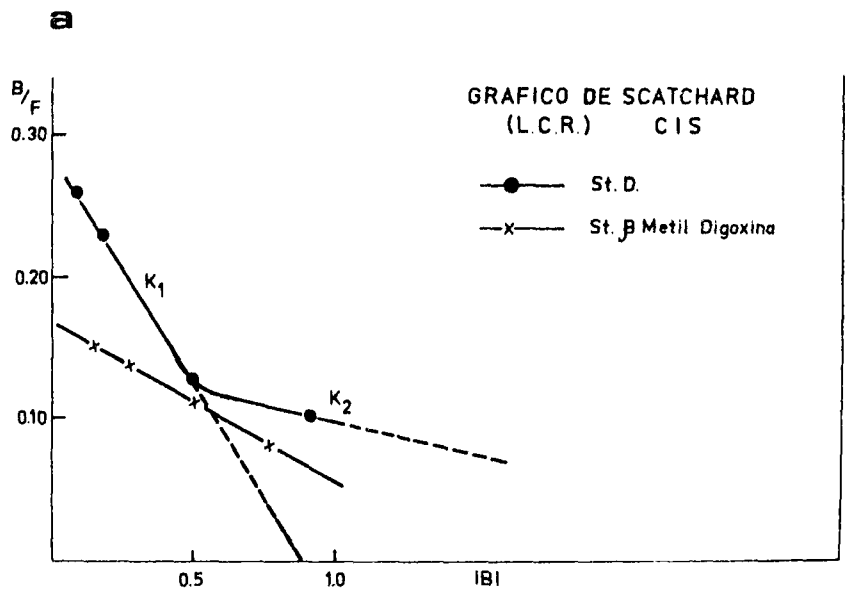


Figure 2


a
B/F
0.30
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0.10
GRAFICO DE SCATCHARD
(L.C.R.) CIS
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St. β Metil Digoxina
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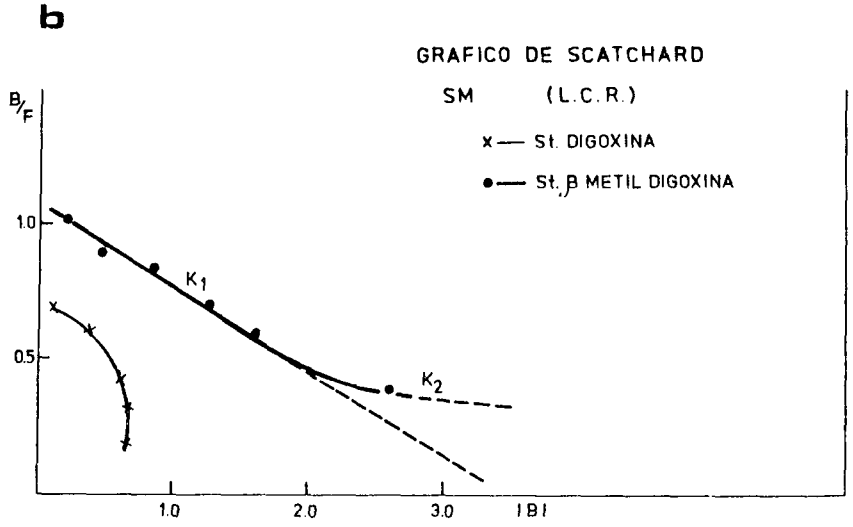
Figure 3


b
GRAFICO DE SCATCHARD
SM (L.C.R.)
St. DIGOXINA
St. β METIL DIGOXINA
B/F
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0.5
K1
K2
1.0
2.0
3.0
IBI

B. GENETICS

B. 1 Genetic Test with Butylated Hydroxitoluene (BHT) and X-Rays in Mature sperm of Drosophila melanogaster

B. Mazar Barnett and E.R. Muñoz.

Butylated hydroxytoluene (BHT) is an anti-oxidant widely used as a preservative in the food industry and its action has been studied in different biological systems with varying results. In Drosophila melanogaster both protecting (1) and sensitizing effects (2) to the genetic damage induced by radiations have been ascribed to the compound. As the published data are scarce and contradictory and the use of BHT is very widespread, a broader study was undertaken of the action of this drug in genetic damage radio-induced in mature Drosophila sperm which includes dominant lethals, sex-linked recessive lethals and II-III translocations.

In all cases wild type seven day Samarkand males were used and the irradiation dose was 2000 Rads of X rays (150 Kv, 10 mA, 1 mm Al). In the combined treatments, a 0.05% BHT solution in 0.4% Na Cl, was administered by intra-abdominal injection, immediately before irradiation. For the detection of recessive lethals the treated males were mass mated for 3.30 hours (which ensures exclusive sampling of sperm, which at the time of treatment was mature and motile) with 5 day "Basc" virgin females. For the detection of II-III translocations the males were mass mated during the same period of time with 5 day cn bw; e females. For the detection of dominant lethals the males were mated individually with cn bw; e females, and immediately afterwards, the females were placed in oviposition chambers for 24 hours. The hatching was controlled 24 and 48 hours later to ensure detection of late hatching eggs. The frequencies obtained in the treated series were corrected for control values with the formula used by Sankaranarayanan (3) and a significance test adapted to normal and binomial distributions was carried out.

The results obtained in the induction of recessive lethals and translocations are shown in Table I and the dominant lethal results figure in Table II. In every case the results of the various experiments have been pooled as there was no significant variation in the frequencies. From the data presented it is evident that in the conditions previously mentioned, in which the experiments were carried out, BHT does not modify the effect of the X rays in respect of any of the genetic damage analysed.

The different methodology (cell sampling, stocks, etc.) does not appear to justify in itself the difference between the data presented here and the potent radiosensitizing action of BHT reported by Prasad and Kamra (2) who, with a much more diluted solution (0.001%) obtained an increase in the frequency of recessive lethals from 5.5% with 2.4 Krads to 9.1% in the combined treatments, a frequency this, higher than that obtained by the same authors with 3.6 Krads (8.8%) i.e. a dose 1.2 Krads higher. There is also a discrepancy with the data published by Guzmân et al. (1) who found that BHT is a radioprotector. In fact, the frequency of 3.06% recessive lethals found by these authors with 2.500 Rads, dropped to 0.70% in combined treatments. The magnitude of the effect is evident if it is borne in mind that the relation between the type of damage studied and the dose is linear.

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TABLE I

Frequencies of sex-linked recessive lethals and II-III translocations induced by 2000 Rads and BHT (0.05%) + 2000 Rads

Treatment	Sex-linked lethal test			II-III translocation test		
	Chrom. tested	Number of lethals	%	Chrom. tested	Number of transl.	%
2000 Rads	1146	74	6.45	1782	77	4.32
BHT + 2000 Rads	1117	73	6.53	1521	62	4.07

TABLE II

Frequency of dominant lethals induced by 2000 Rads and BHT (0.05%) + 2000 Rads

	Control			2000 Rads			BHT + 2000 Rads		
	N° eggs laid	N° eggs non hatched	%	N° eggs laid	N° eggs non hatched	%*	N° eggs laid	N° eggs non hatched	%*
Series I	511	63	12.33	1220	551	37.45	770	365	40.00
Series II	1856	243	13.09	2067	1127	47.67	1913	1049	48.04
Total	2367	306	12.93	3287	1678	43.78	2683	1414	45.68

* Corrected with the formula $\frac{Pt - Pc}{1 - Pc}$ (3). Pt: proportion of unhatched eggs in the treatment; Pc: proportion of unhatched eggs in the control.

B. 2 Sex-Linked Recessive Lethal Tests with 1-Benzyl-3-(2,3 Dihydroxypropoxy) -Indazole and 2N-Butyl-Malon Di (Dimethyl) Amine-propyl) Diamide in Drosophila melanogaster

B. Mazar Barnett and E.R. Muñoz

The increasing number of new products launched to the market by the chemical and pharmaceutical industry demands verification of their potentical mutagenicity in different systems. This is extremely important both for the industry itself and for the consumers in view of growing concern about the increase of genetic risk to which environmental pollutants in general may give rise. The importance of negative or positive results in this field is obvious.

The possible mutagenic effect of two substances of marked antiinflammatory, analgesic and antipyretic action recently synthetized by a local pharmaceutical laboratory: 1 Benzyl-3-(2,3 dihydroxypropoxy)-indazole (1) and 2n-butyl-malon di (dimethyl-amine-propyl) diamide was studied in Drosophila melanogaster. For this purpose sex-linked recessive lethality tests were carried out, which is one of the most effective methods of detecting mutagenic activity in Drosophila (2).

The 1-benzyl-3-(2,3 dihydroxypropoxy)-indazole was administered orally to 7 day Samarkand males by means of filter papers saturated with the drug in Tween 20 and glucose, over 24 and 48 hour periods. It could not be injected intra-abdominaly (the ideal way to ensure the incorporation of the drug and its accurate dosage) owing to the insolubility of the available lyophilized sodium succinate. After treatment the males were mass mated with "Basc" five day virgin females for 24 hours to study sperm treated in the final stages of development. 5 subcultures of two days duration were made and with the F1 females individual matings were carried out in order to detect sex-linked recessive lethal mutations.

A 3.75% solution adjusted to pH 8.5 with ClH 1N of 2n-butyl-malon di (dimethyl amine propyl) diamide was administered by intraabdominal injection. The males treated were mass mated with "Basc" virgin females and were provided with new females every 24 hours with the purpose of studying cells which at the time of treatment were at different stages of development. The lethality tests were carried out with females fertilized on the first, second and fifty day.

Since the frequencies of lethals obtained in the various subcultures and sucessive broods did not differ, the data was pooled. The results are shown in Table I. In the experimental conditions described the lethal mutation rates shown by the treated series, do not differ significantly from the controls, which exhibited the usual spontaneous lethal frequency for the stock used.

Acknowledgement

The authors thank Laboratorios Szabo Hnos., Kessler y Cía. S.R.L. for providing the drugs.

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TABLE I

Sex-linked recessive lethal tests in Drosophila melanogaster with 1-benzyl-3-(2,3-dihydroxypropoxy)-indazole and 2N-butyl-malon di (dimethylamine propyl)-diamide

Treatment	Chromosomes tested	Number of letals	% of lethals
Drug I	11,207	22	0.20
Drug II	6,328	15	0.24
Control	1,464	4	0.27

B. 3 The Influence of the Impairment of Egg Laying on Dominant Lethality in Drosophila melanogaster

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Some radiomimetic (alkylating) agents, previously believed not to be efficient chromosome breakers, are in fact quite active, although for this action to appear the treated cells must be stored for variable periods (storage effect). The usual way to demonstrate this effect is to treat sperm, either in the males or in inseminated females, which are then kept in a protein deficient (agar 4% - glucose 2%) medium, to avoid egg-laying. After the storage the females are transferred to normal food for the genetic tests.

A connection has been suggested between inadequate nutrition of females and "storage effect", since the lack of protein could alter the repair mechanisms of genetic damage. Attempts made to determine whether the degree of dominant lethality induced by EMS varied when the females were stored in normal or deficient medium are not conclusive (1).

When studying storage effect, other factors must also be considered e.g. aging of sperm and eggs when the females are stored in media that discourage oviposition. Aging of sperm does not seem to alter the final frequency of damage recovered (2). To study the influence of egg retention on the frequency of spontaneous dominant lethals, 5 day old Oregon K impregnated females were kept for 5 days in ordinary medium, at 17°C, and in deficient (agar) medium at 25°C. They were then transferred to oviposition chambers with whole medium, at 25°C and the eggs of two successive 24 hour each egg laying periods were scored. 24 and 48 hours later the hatched and non-hatched eggs were scored to detect late hatching.

Similar tests were carried out with 5 day old virgin females kept in ordinary food and transferred to oviposition chambers immediately after mating. Table I shows that the frequency of non hatched eggs is significantly higher when the females are stored in deficient (agar) medium. In this group, the frequency of lethals in the first laying period is significantly higher than in the second, which does not happen with the females stored in ordinary medium. Table II shows three experimental series of non-stored females, and it can be seen that the frequency of lethals in the first egg laying period is always significantly higher than that in the second. From the analysis of the data it is evident that, irrespective of the influence of the nutritional state of the females, the frequency of dominant lethals is affected by egg storage. Table I shows that females stored in deficient medium retain the eggs, which are then unable to develop normally. In the second laying period many of the mature and witheld eggs have already been eliminated, and ther is a drop in the frequency of lethals. Owing to the short time elapsed between the two oviposition periods and to the advanced stage of development of the eggs, it seems unlikely that this decrease is caused by the richer food in the oviposition chambers.

A completely different picture is shown in the experiments with normal food; the females do not retain their eggs which they lay during the whole storage time. This is still more evident in Table II where, in spite of the females having been properly fed before mating, a higher lethality can be seen in the first laying period, since this period includes those eggs normally retained by the females before they are fertilized. It is evident that when studying the influence of storage on chromosome breaks and point mutations, the physiological alterations that stored eggs undergo must be taken into account (the extreme case being death of the eggs), since these alterations may affect in different ways the recovery of the various types of genetic damage induced.

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TABLE I

Frequency of dominant lethals in Drosophila melanogaster females stored during five days

	A) Deficient medium			B) Complete medium		
	Total eggs	Unhatched eggs	%	Total eggs	Unhatched eggs	%
1st. laying period	552	235	42.57	843	39	4.63
			p<0.0001			p=0.08
2nd. laying period	654	137	20.97	449	31	6.90
Total	1206	372	30.85	1292	70	5.42

Total A) vs. Total B) : p<0.0001

TABLE II

Frequency of dominant lethals in non-stored Drosophila melanogaster females

	Total eggs	Unhatched eggs	%	p
I				
1st. laying period	1414	93	6.58	0.00010
2nd. laying period	577	13	2.25	
II				
1st. laying period	745	78	10.47	<0.00010
2nd. laying period	440	13	2.95	
III				
1st. laying period	1194	88	7.37	<0.00010
2nd. laying period	742	14	1.89	
Total				
1st. laying period	3353	259	7.72	<0.00010
2nd. laying period	1759	40	2.27	

B. 4 The Influence of Temperature on the Recovery of Genetic Damage Induced by X-Rays in Drosophila melanogaster Males

B. Mazar Barnett.

With the purpose of obtaining information on the nature of radioinduced genetic damage and its repair, it was considered of interest to study the effect of low temperature on irradiates sperm of Drosophila melanogaster. The investigation was restricted to late spermatids and motile sperm because their well established differences in radiosensitivity (2, 5, 3) provides an excellent system for studying the influence of external agents on the effects of radiation.

The effects of a low temperature (0°C) and X radiation on the induction of sex-linked recessive lethal mutations and II-III translocations were investigated.

The experimental procedure was as follows: irradiation was carried out at 10 mA, 150 kV, 1mm Al. 1000 R and 2000 R were delivered from a target distance of 26 cm. Double walled polystyrene containers filled with a mixture of crushed ice, and a solution of NH_4Cl were used to keep the flies at 0°C. (This was replaced with rice for the irradiations at room temperature). The flies were subjected to the low temperature 5 minutes before the irradiation and kept at 0°C for 40 minutes including the time of irradiation. Two methods were used to study the two stages of sperm development with coincident results. The impregnated females were subcultured at 4 day intervals for 12 days. Sex-linked recessive lethal tests were done with treated Samarkand wild type males and Basc females. The results are shown in Table I. II-III translocation tests were carried out with treated Samarkand males and cn bw; e females. The results are shown in Table II. From the data obtained, it follows that: a) At room temperature, motile sperm is more radiosensitive than late spermatids with respect to the two parameters considered, and at 0°C the frequency of recessive lethals observed in late spermatids increases significantly over that induced at room temperature, raising the level of lethals to the frequency shown by motile sperm. The latter is not modified by the low temperature.

b) The low temperature treatments result in an increase in the frequency of II-III translocations in both cell stages.

If the lower radiosensitivity of late spermatids with respect to motile sperm at room temperature can be ascribed to repair of premutational damage, it might be possible that at 0°C, the process is impaired. In this case the absence of repair of premutational damage in fully mature sperm would account for the lack of effect of the low temperature at this cell stage. On the other hand it can be said that low temperature does not cause an increase in the radiation damage recovered from motile sperm.

Since the recovery of translocations implies two processes: breakage of chromosomes and healing of fragments, the outcome of these experiments suggests a mechanism pertaining either to the enhancement of radiation damage or to repair processes, common to both cell stages. If at room temperature a certain amount of restitution of breaks, or potential breaks, takes place, it may be supposed that the low temperature impairs this restitution. An increased number of fragments would then be available later for misrepair, with the consequent increase in the number of translocations recovered.

The results obtained seem to support the hypotesis that the low temperature acts by impairing the normal functioning of the enzymes involved in the processes

of genetic repair. Nevertheless, the possibility of an enhancement of radiation damage brought about by an increase in cellular oxygen concentration, can not be ruled out (1, 4).

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TABLE I
Sex-linked recessive lethals induced by 1000 R of X rays in Drosophila melanogaster males

	Room temperature			0°C		
	N° chrom. analyzed	N° of lethals	% of lethals	N°chrom. analyzed	N° of lethals	% of lethals
Motile sperm (1st. mat.perm.:3.30 h)	1579	44	2.78	1614	37	2.29
	1420	34	2.39	951	27	2.84
Motile sperm (1 observed mating)	2943	74	2.51	1708	45	2.63
Total	5942	152	2.56	4273	109	2.55
Late spermatids (2nd. mat. per. up to 24 h)	2237	34	1.51	1430	32	2.24
	1899	32	1.68	1227	33	2.69
Late spermatids from 2 h old males)	1571	24	1.53	1140	28	2.46
Total	5707	90	1.58 p 0.0020	3797	93	2.45

TABLE II

II-III translocations induced by 2000 R of X rays in *Drosophila melanogaster* males

	1st. mating period (motile sperm)				2nd. mating period (late spermatids)			
	N° chrom. analyzed	N° of transl.	% of transl.	p	N° chrom. analyzed	N° of transl.	% of transl.	p
Irrad. room temperature	1867	87	4.66		2384	59	2.47	
	1782	77	4.32		1573	49	3.12	
	1365	51	3.74		1362	37	2.72	
Total	5014	215	4.29		5319	145	2.73	
				0.00006				0.00006
Irrad. 0°C	917	56	6.11		1676	72	4.30	
	1204	85	7.06		1517	63	4.15	
	1973	128	6.49		1725	87	5.04	
Total	4094	269	6.57		4918	222	4.51	

	24 h mating period			Average from the 2 mating periods		
Irrad. room temperature	1981	62	3.13	10,333	360	3.48
Irrad. 0°C	2614	127	4.85	9,012	491	5.45

B. 5 Preliminary Data on the Reversion of the Alcohol Dehydrogenase Locus in Drosophila melanogaster

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It is of interest to determine the true nature of the genetic damage induced by ionizing radiations in Drosophila melanogaster. Due to the difficulties inherent in the fine mapping of chromosomes in this organism, it is not always possible to distinguish between gene mutations and small chromosome deficiencies. Moreover, the induction of reversions, currently used in organisms such as bacteria and Neurospora sp. for that purpose, is difficult to apply in Drosophila because of the great number of individuals which must be tested to reach valid conclusions. Reversion frequency has been determined in some loci which are considered particularly unstable: Killer-prune, 1×10^{-4}, induced with 3000 R (4); forked-3n, 6.1×10^{-4} induced with 4000 R (5); yellow-2, 6.24×10^{-5}; scute, 6.01×10^{-5}, White apricot, 2.09×10^{-5} induced with 5000 R (2); Nasobemia, 7×10^{-4} with 4000 R (1). Based on the fact that mutant flies deficient in the enzyme alcohol dehydrogenase (Adh) in Drosophila melanogaster are selectively killed when the culture medium contains ethanol, a method has been developed for the detection of reversion of this mutation.

This renders possible the testing of a great number of individuals in a relatively simple manner. The study of reversions of Adh mutants with this method was undertaken by means of X rays and diethyl sulphate.

The experimental procedure is as follows: adult males of a stock deficient in the enzyme, (Adh-n1, induced by ethyl methane sulphonate (3)) are treated with a mutagenic agent and mated with virgin females also deficient in the enzyme and bearing genetic markers which allow the chromosome under study to be followed up. (In these experiments the mating period was 24 hours). The F1 flies are placed in a medium made of agar 23% and ethanol 17.5% for 24 hours, after which the results are checked. Optimun ethanol concentration, that in preliminary tests killed the adults homozygous for the Adh mutation in a few hours, but did not kill normal flies nor heterozygotes for the mutation. This means that in those cases in which mutations are reversed, the individuals treated with alcohol should survive. Two series of experiments were carried out, with their respective controls: a) males treated with 1000 R of X rays, with a total F1 progeny of 292,980 tested and 77,930 in the control; b) males treated orally with 0.5% diethyl sulphate, with a total F1 progeny of 158,000 tested and 137,850 in the control. For the purpose of checking whether the males had ingested the drug, a sex linked recessive lethality test was carried out with part of those treated. The rate of induced mutations reached 31.27%.

No reversions were detected in the locus studied, which shows that the reversion frequency in the case of treatments with X rays would be below 3.4×10^{-6} and in the case of treatment with Diethyl sulphate below 6.30×10^{-6}. It follows that no suppressors of the mutation under study were induced. The experiments are continuing and exposure to radiation at 1000 R will be kept up, since larger doses induce a great number of chromosome breaks which tend to confuse the results.

The authors gratefully acknowledge Dr. E.H. Grell for the Adh stocks.

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B. 6 Translocation Frequency Induced by X-Rays in Drosophila melanogaster Males Pre-Treated with Chloramphenicol

E.R. Muñoz.

As induced genetic changes go through a series of steps as a prior condition to their final fixation, agents capable of altering this process may determine variations in the amount of damage recovered. By using antibiotics, it has been possibly to modify the genetic effect induced by ionizing radiations in various organisms. In Drosophila melanogaster pre-treatment with chloramphenicol may reduce the frequency of sex-linked recessive lethals induced by X-rays in spermatids (1, 4) and possibly in spermatomacytes (1). The results in sperm are contradictory in relation to the induction of this type of damage and as, in the above mentioned tests a ring X chromosome was used, lethals connected with chromosome aberrations were excluded.

Since the influence of chloramphenicol on the processes of rejoining of chromosome fragments induced by radiation in Drosophila has not yet been established, a translocation test between chromosomes II and III was carried out with males treated with this antibiotic before irradiation.

Wild type 7 day Oregon R males were injected intraabdominally with a solution of chloramphenicol (0.05 mg/ml) and after 40 minutes were irradiated with a dose of 3000 Rads of X rays (150 kV, 10 mA, 1 mm Al), non-injected males were irradiated simultaneously. Immediately after the treatment males were mass mated (in a proportion of 1 to 3) with 5 day virgin females homozygous for the cn bw; e markers for 24 hours. With the F1 males, translocation tests between chromosomes II and III were carried out and since aberrations may adversely affect fertility, this was also scored.

Table I shows the translocation and sterility frequencies obtained and it can be seen that pre-treatment with chloramphenicol leads to a significant decrease in damage.

Owing to the fact that between the induction of genetic damage and its detection, processes take place that require protein synthesis and that chloramphenicol is an inhibitor of this synthesis, the variations in the frequency of damages found with this antibiotic in other organisms, have been interpreted as being due to interferences with these processes. In Drosophila, results with fractioned radiation doses would seem to indicate that the chromosome fragments induced in the cells under study (mature sperm and late spermatids) interact after fertilization (2, 3). However, if the reduction in translocations found in the treatment with chloramphenicol is due to protein synthesis impairment, these results would indicate that some fragments rejoin before fertilization. Another possibility is that under the conditions in which the experiment was carried out, this drug acts by disturbing more general metabolic processes, which by rendering more difficult the subsequent rejoining of the chromosome fragments, would lead to their selective elimination, thus reducing the amount of final damage recovered. Our present knowledge of genetic repair mechanisms in Drosophila, does not allow the exclusion of either hypothesis.

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TABLE I

Frequencies of II-III translocations and male sterility induced by 3000 Rads of X-rays with and without chloramphenicol pre-treatment (0.05 mg/ml) in *Drosophila melanogaster*

Treatment	II-III Translocations				Sterile males in the F1			
	Number tested	N° of trans.	%	p	Number tested	N° of ster.	%	p
Control	2706	-	-		2757	51	1.84	
3000 Rads	1358	192	14.13	0.036	1725	367	21.27	<0.0001
Chloram. + 3000 Rads	871	96	11.02		1025	154	15.02	

B. 7 Dominant Lethals Induced by Diethyl Sulphate in Drosophila melanogaster

E.R. Muñoz and B. Mazar Barnett.

No translocations having been found in Drosophila melanogaster after treatment with Diethyl sulphate (DES), it is considered that this drug does not induce chromosome breaks in this organism (3).

With the purpose of establishing whether the lack of translocations in really due to DES incapacity to induce breaks or to problems related to their detection in Drosophila, dominant lethality tests were carried out, since this damage is largely the expression of chromosome breakage and the resulting complex aberrations.

Wild type 7 day Samarkand males were treated orally with 0.5% and 0.75% solutions of DES during variable periods (see tables) and were then mated individually with virgin females of the same stock. With the purpose of exclusively studying sperm which at the time of treatment was mature and motile, after a single mating the males were discarded and the females placed in oviposition chambers at 25°C. The females which after 24 hours had laid more than 20 eggs were transferred to another chamber for a second oviposition period.

As the majority of induced dominant lethals act in early embryogenesis, the hatchability of the eggs was determined (48 hours were allowed for scoring, to ensure detection of late hatching).

The results of these early dominant lethality tests are shown in Table I. As may be seen, the early dominant lethality rates in the treated series are significantly higher than those shown by the controls. The difference found in some series between the first and second egg laying period will be discussed elsewhere. To establish a comparison it should be pointed out that the frequency of early dominant lethals induced by a solution of 0.75% DES for 2 1/2 hours, is similar to that obtained with 2000 Rads of X-rays (43.78%). This dose induced 4.32% II-III translocations.

Given the high toxicity of DES and for the purpose of discarding the possibility that the effect observed might have been due to physiological alterations of the sperm, a study was made of the action of this drug on the mortality of larvae and pupae (late dominant lethality), whose father had been treated. This is justified by the fact that the genetic damage induced by chemical substances in Drosophila, frequently remains latent for many cell generations and appears after considerable delay as chromosome breaks (2).

Hatched eggs and adults were scored, and the results can be seen in Table II, there being a significant increase in the late dominant lethality in the series treated in relation to the control.

These results are taken as evidence that DES induces chromosome breaks in Drosophila. The lack of detections of translocations (3) found, might be due to: a) breaks induced by this chemical cannot interact and hence the lack of translocations and the high frequency of dominant lethals or b) DES induced breaks remain latent and become effective after several cell generations leading to the death of the heterozygote before it reaches the adult stage. The latter would seem more probable in view of the results obtained with another monofunctional alkylating agent, Ethyleneimine, that induces translocations in Drosophila sperm with a frequency proportional to its storage in the female (1, 4). This would allow the realization of breaks and the interaction of broken ends. Experiments along these lines are being carried out with DES.

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TABLE I

Early dominant lethality induced by diethyl sulphate in mature sperm of Drosophila melanogaster

Treatment	Control		Series I		Series II	
DES 0.5%	Unhatched eggs / Total eggs	% Dom. Leth.	Unhatched eggs / Total eggs	%* Dom. Leth.	Unhatched eggs / Total eggs	%* Dom. Leth.
1st. laying period	93 / 1414	6.58	481 / 1614	24.86	406 / 1044	34.59
2ad. laying period	13 / 577	2.25	212 / 814	24.34	224 / 757	27.97
Total	106 / 1991	5.32	693 / 2428	24.52	630 / 1801	31.33
DES 0.75%						
1st. laying period	90 / 1228	7.33	726 / 1232	55.68	498 / 660	73.51
2nd. laying period	13 / 734	1.77	136 / 276	48.37	223 / 247	90.10
Total	103 / 1962	5.25	862 / 1508	54.79	721 / 907	78.35

DES 0.5% : Duration of treatment: Series I, 2.30 h; Series II: 3 h.

DES 0.75%: Duration of treatment: Series I, 2.30 h; Series II: 2.30 + 1 h (1 h interval).

* % correction: $\frac{Pt - Pc.}{1 - Pc}$ Pt: proportion of non hatched eggs in the treatment; Pc: proportion of non hatched eggs in the controls.

TABLE II

Late and total dominant lethality induced by diethyl sulphate (0.75%) in mature sperm of Drosophila melanogaster

Egg laying period	Proportion of late dom. leth.* x 100***			Proportion of total dom. leth.** x 100***		
	Control	Series I	Series II	Control	Series I	Series II
1st.	$\frac{24}{1138} = 2.11$	$\frac{73}{506} = 12.59$	$\frac{21}{162} = 11.08$	$\frac{114}{1228} = 9.28$	$\frac{799}{1232} = 61.25$	$\frac{519}{660} = 76.46$
2nd.	$\frac{21}{721} = 2.91$	$\frac{18}{140} = 10.25$	$\frac{5}{24} = 18.45$	$\frac{34}{734} = 4.63$	$\frac{154}{276} = 53.65$	$\frac{228}{247} = 91.94$
Total	$\frac{45}{1859} = 2.42$	$\frac{91}{646} = 11.96$	$\frac{26}{186} = 11.85$	$\frac{148}{1962} = 7.54$	$\frac{953}{1508} = 60.21$	$\frac{747}{907} = 80.93$

* Late dom. Lethality : $\frac{\text{N° hatched eggs - N° adults}}{\text{N° batched eggs}}$

** Total dom. lethality: $\frac{\text{Early dom. leth.} + \text{Late dom. leth.}}{\text{Total N° of eggs}}$

*** % corrected: $\frac{Pt - Pc.}{1 - Pc}$ Pt: proportion of treated; Pc: proportion of controls

Series I: duration of treatment: 2.30 h.

Serie II: duration of treatment: 2,30 h + 1 h (1 h interval).

C. IMMUNOLOGY

C.1 Anti-Allogeneic Graft Reaction in Diffusion Chambers. I. Evidence of Cell Destruction

E.E. Capalbo.

By assuring the depression of the circulating agglutinin titer produced by immunologically component mouse spleen cells of a paternal P_2 strain cultivated "in vivo" in X-irradiated, immunologically inert, hybrid $P_1 \times P_2$ mice, Celada and Carter (1) evaluated the killing effect of mouse spleen cells from a paternal P_1 strain previously sensitized to P_2 antigens. With this technique they could observe a decrease in the antibody titer comparable to an almost complete rejection of the inoculated P_2 spleen cells. They suggested that this result could be due to cell destruction or inhibition of protein synthesis or both. In the present study the first possibility was investigated using the diffusion chamber culture technique and mouse spleen cells. Each diffusion chamber of 0.1 micron membrane porosity was inoculated with 24×10^6 C3H spleen cells from a donor previously sensitized to C57B1 antigens, plus 24×10^6 C57B1 spleen cells from a donor sensitized to rat antigens (rat red blood cells-RBC) plus rat RBC. Suitable control groups were also utilized. In one experiment the donors of the C3H cells were labeled "in vivo" by an intraperitoneal injection of 5 µCi tritiated thymidine. In a second experiment the donors of the C57B1 cells were labeled in the same manner as the C3H donors.

At varying intervals after peritoneal implantation of the chambers the number of labeled lymphoid cells and the average number of grains per labeled cell was determined by means of radioautography with Kodak nuclear track emulsion NTB 3.

The sealed chambers, one per mouse, were intraperitoneally implanted into sublethally 500 r X-irradiated C3H mice.

These two findings would indicate that the inhibition of the proliferation of allogeneic labeled cells and they disappereance could be due to a direct effect of the pre-sensitized C3H cells, which instead of committing suicide proliferate at a higher rate resembling an immunologic reaction similar to a secondary antibody response.

1.- Celada, F. and Carter, R., J. immunol., 89:161, 1961.

2.- Capalbo, E.E. et al., J. Immunol., 92:243, 1964.

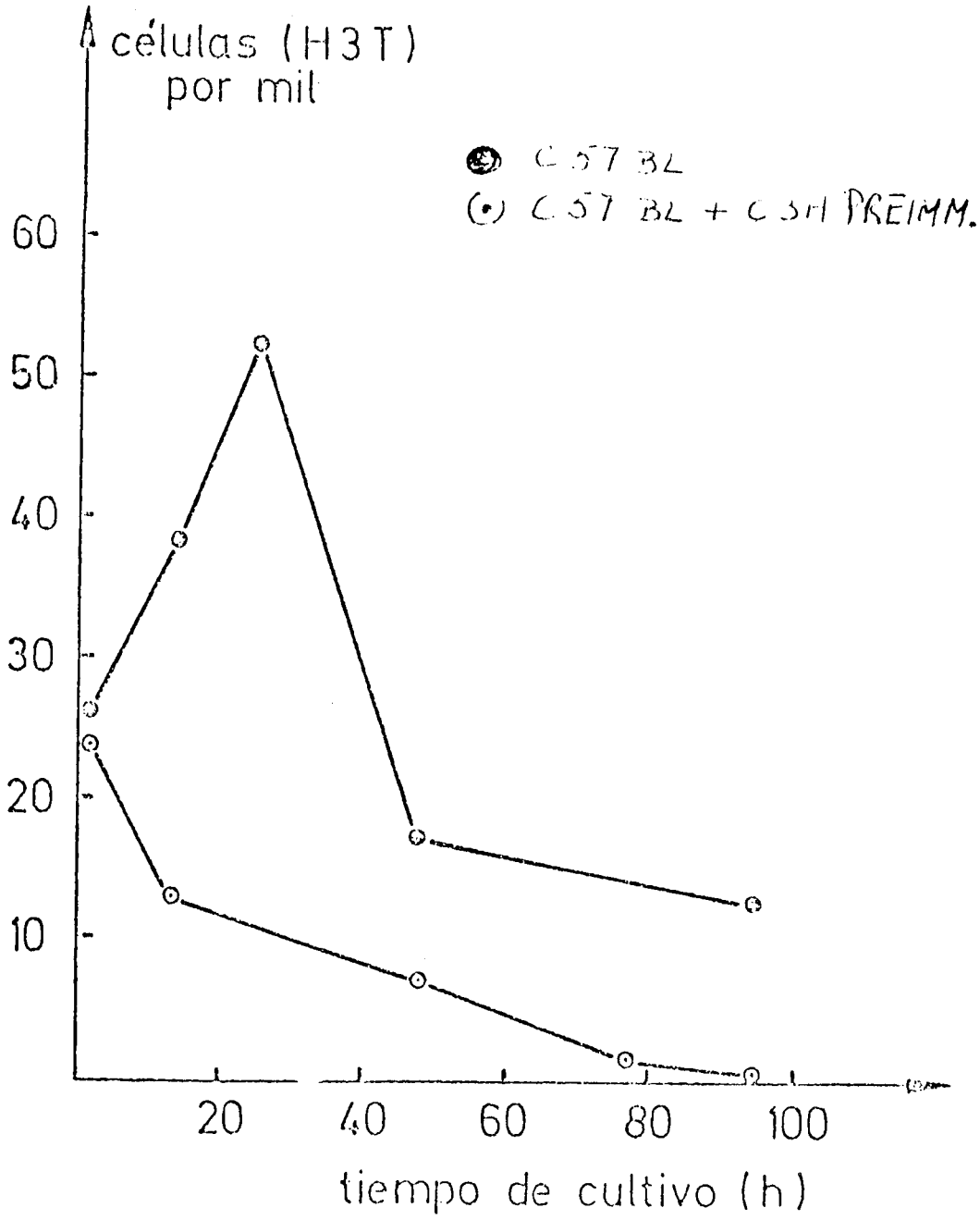

Figure 1.- Ilustrates the duplication time of labeled C57B1 spleen cells cultured in the presence or in the absence of C3H spleen cells from donors sensitized with C57B1 antigens. When cultured in the absence of pre-sensitized C3H cells (uuper curve) C57B1 cells (target cells, allogeneic antigen) proliferate normally with a duplication time of about 24 hrs, while when these cells are cultured together with pre-sensitized C3H cells, their proliferative capacity is highly depressed and the number of labeled cells decreases progressively and significantly during the first 48 hours of the experiment.

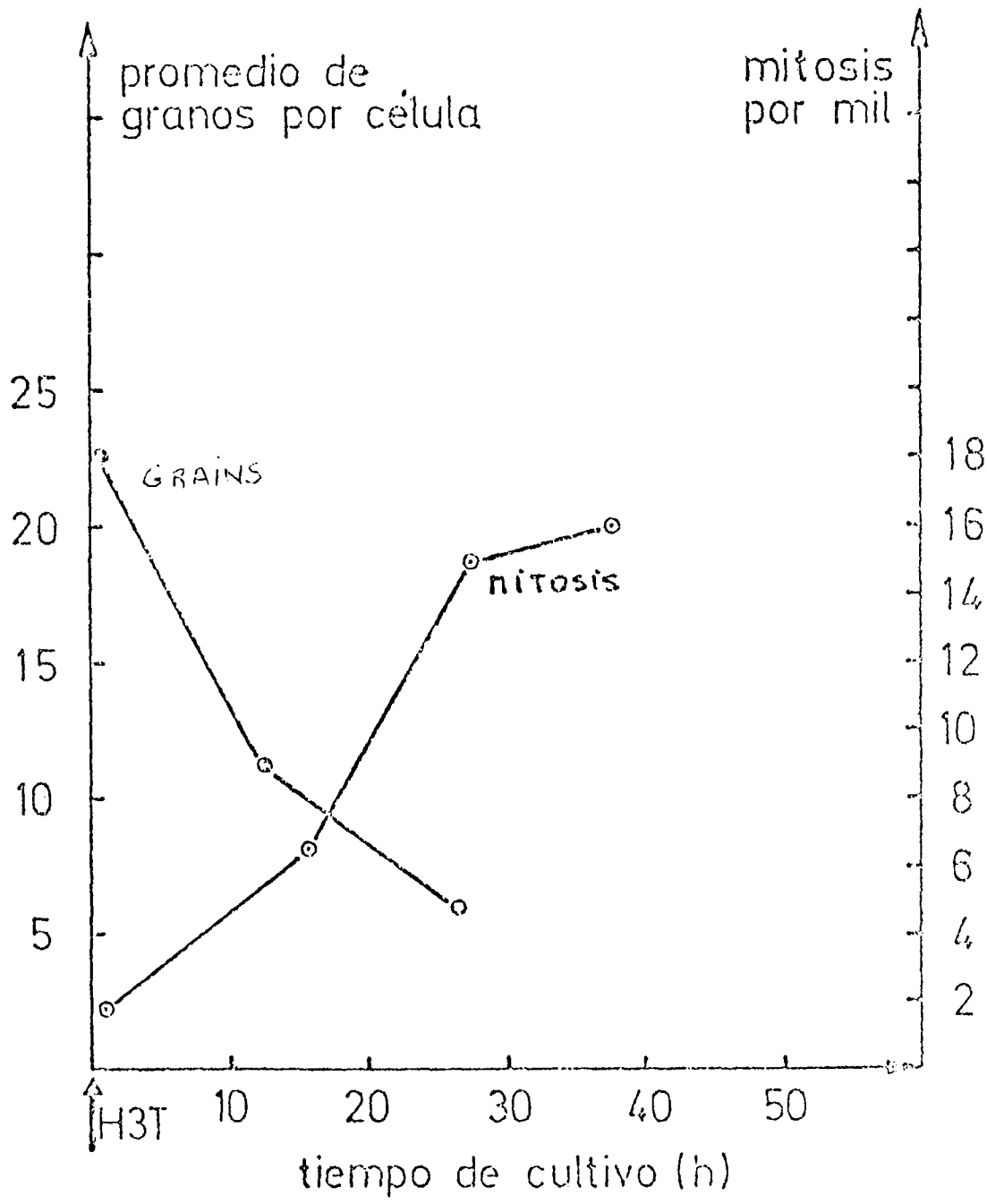

Figure 2.- Ilustrates the average number of silver grains and the mitotic index of C3H cells pre-sensitized with C57B1 antigens during 40 hours of culture with C57B1 spleen cells. As can be seen the average number of grains per labeled lymphoid cell decreases progressively indicating for this type of cells a duplication time of about 12 hours. Acordingly with this decrease in the number of grains per cell the number of mitosis increased (duplication time approximately 12 hours).

C. 2 Anti-Allogeneic Graft Reaction in Diffusion Chambers.

II. Evidence of the Existence of a Humoral Factor.

E.E. Capalbo.

In previous experiments using the diffusion chamber cell culture technique, tritiated thymidine labeling and radioautography it was observed that during the allogeneic graft reaction target allogeneic cells are destroyed between 24-28 hs. This cell destruction was probably due to an immnunological process mediated by a sesil antibody. To investigate the simultaneous occurrence of a humoral anti-allogeneic antibody, several experiments were performed with spleen cells from mouse strains differing in the H-2 locus of histocompatibility genes.

In these experiments the diffusion chamber technique was modified in order to allow both populations of killer and target cells to be cultured in the same recipient but separated from each other, in order to avoid cell-to-cell contact. In order to achieve this objective diffusion chambers of double compartment separated by a 0.1 micron porosity Millipore membrane were built with the same materials.

In one of the compartments 24×10^6 spleen cells from C3H (H-2 kk) mouse immunized 2 or 7 days before with C57B1 (H-2 bb) antigens were innoculated; and in the second 24×10^6 C57B1 spleen cells were innoculated.

Both cell populations were labeled by means of an intraperitoneal injection of 5 µCi of tritiated Thymidine in the sublethally X-irradiated recipient mouse.

By means of radioautography in samples of diffusion chamber fluid taken at various intervals after chamber implantation and radioisotope labeling, the proliferative rate of lymphoid cells of both cell populations during the five days culture was determined. The results can be seen in figure 1 in which the tritium H3 index is plotted in the ordinate against time in abscisae. The proliferation of C57B1 spleen cells (broken line) is highly depressed when these cells are cultured with C3H cells in the opposite comparment, transferred 2 or 7 days after the allogeneic "in the donor" immunization. In the control group without C3H cells (not plotted), C57B1 cells proliferate at a normal rate with duplication time of 24 hours.

In the presensitized C3H cells compartment on the contrary reacting tritium labeled cells proliferate at a higher rate than non treated cells, with a duplication time of about 12 hours (full line). In previous experiments it was demonstrated that the duplication time for C3H untreated cells is 24 hours. This steep inhibition of allogeneic cell proliferation occurring only in the experimental group would indicate the production in the C3H compartment of a humoral, mobile factor which after diffusing through the Millipore membrane reacts with the all membrane determinants of the allogeneic cells.

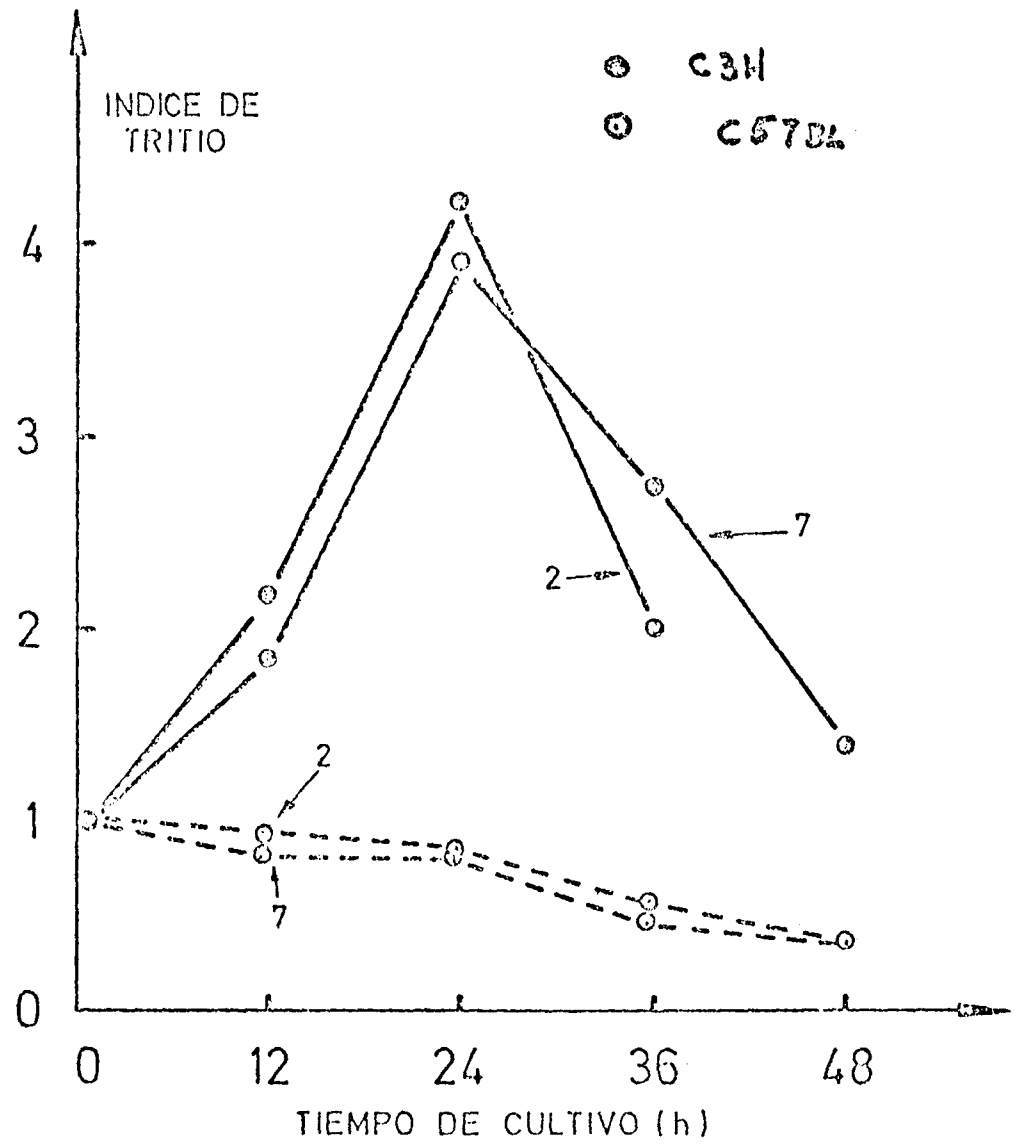
INDICE DE TRITIO
C3H
C57BL
4
3
2
1
0
2
7
2
7
0
12
24
36
48
TIEMPO DE CULTIVO (h)

Figure 1

C.3 Effect of Phytohemagglutinin on Mouse Spleen Lymph Nodes and Thymus

E.E. Capalbo.

Despite the numerous reports on the effect of phytohemagglutinin on nucleated cells of peripheral blood it is not yet clear if the blastogenesis of circulating lymphocytes is due to a direct mitogenic stimulus or to other mechanisms. Among these, the possibility that phytohemagglutinin behaves as an antigen of a strong immunogenic property, and also that blastogenesis occurs through modulation of more or less mature cells without mitosis, are of special interest.

A first approach to investigating if phytohemagglutinin behaves like an antigen, an "in vivo" experiment was performed and results were obtained which indicate its immunogenic nature and that blastogenesis occurs with mitosis.

For this experiment males of the C31F1 mouse strain 10 to 12 weeks of age were used. Aliquots of Difco phytohemagglutinin P diluted in 0.15 M NaCl were intraperitoneally injected into each mouse. Three mice per point were sacrificed by means of a transversal incision of the throat immediately after the injection, and at 24, 48, 72, 96 and 216 hrs. thereafter. At each point, body, spleen, mesenterical lymphnodes and thymus weights were recorded separately. The injection of 0.4 ml of Phytohemagglutinin P produced modifications in the weights of these organs as can be seen in figure number 1. There was a significant increase in the weight of spleen and lymph nodes 72 hours after the Phyto-P injection, this being more accentuated in the former. As can be seen in figure 2 where the values are plotted as a percentage of the initial value, it was an increment of 175.2 % and of 151 % in weights of the spleen and in the lymph nodes respectively. Regarding the modifications on the thymus weight a significant decrease was observed, suggesting an accentuated thymus cell migration to other lymphoid organs. Moreover in cytological preparations of spleen and lymph node smears a significant increase in the number of mitosis per 1000 cells (from 0.01 to 1.0) at 12-24 hours compared with the untreated control was observed.

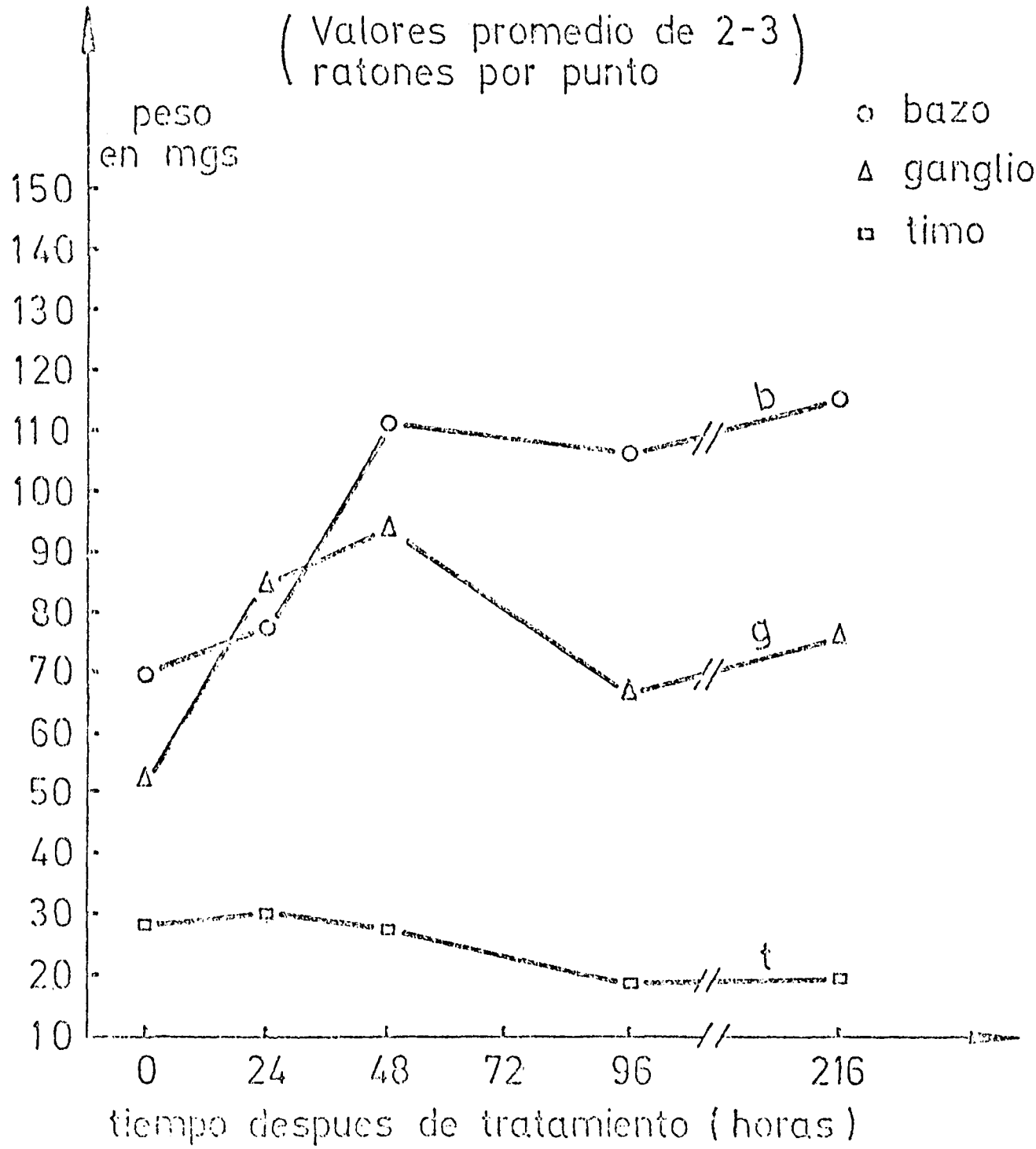
(Valores promedio de 2-3 ratones por punto)
peso en mgs
o bazo
Δ ganglio
□ timo
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
b
g
t
0
24
48
72
96
216
tiempo despues de tratamiento (horas)

Figure 1

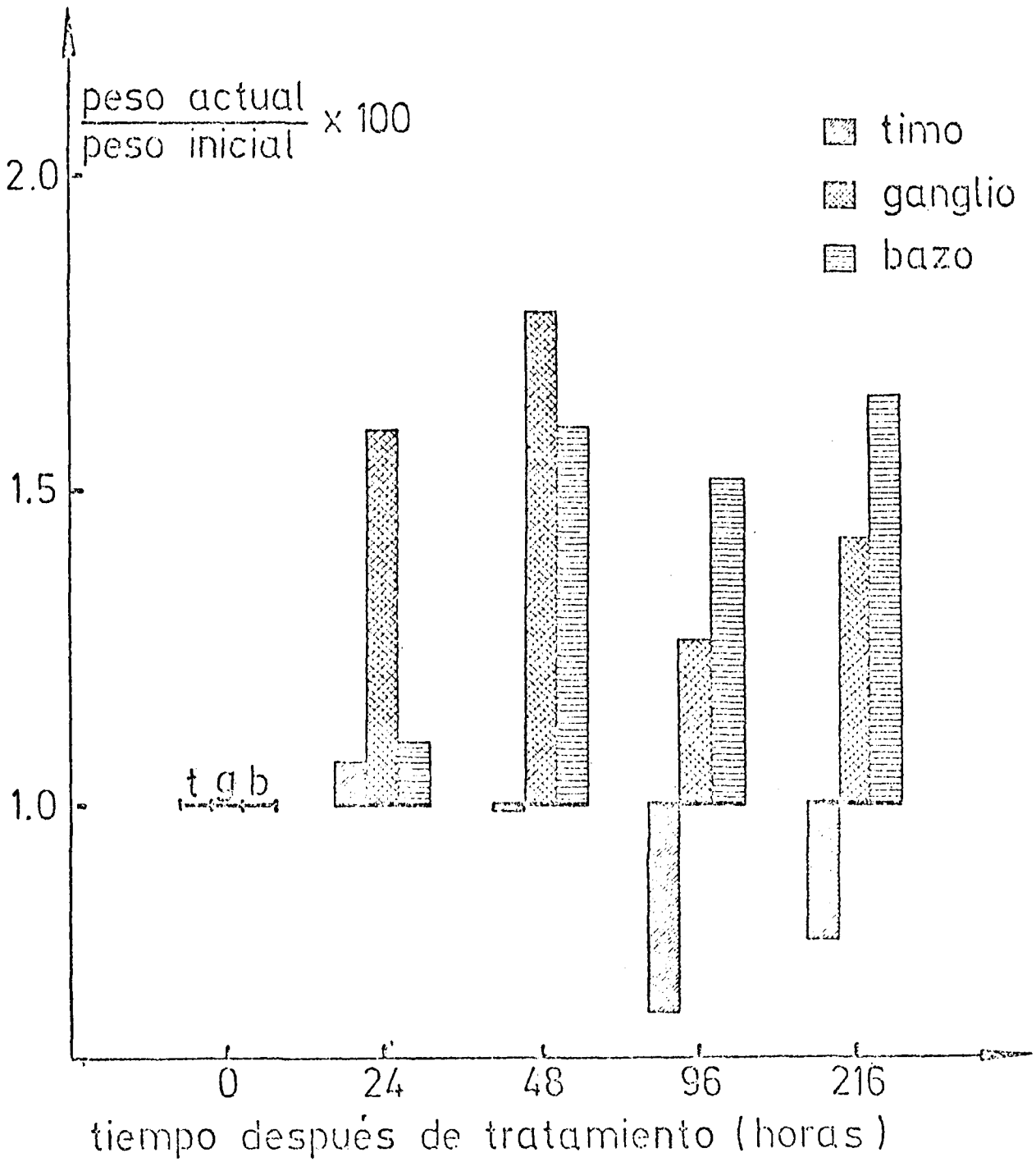
peso actual / peso inicial x 100
timo
ganglio
bazo
2.0
1.5
1.0
t g b
0
24
48
96
216
tiempo después de tratamiento (horas)

Figure 2

C. 4 Familial Goiter with Partial Iodine Incorporation Block and Euthyroidism Due to the Deficient Peroxidase Defect

H. Niepominiszcze,* O.J. Degrossi,* L.M. Scavini and H.P. Curutchet*

Several abnormalities, at the level of thyroid peroxidase, have been shown to be a cause of congenital goiter with iodine incorporation defects (1-3). One of them, the so-called deficient peroxidase defect seems to be a quantitative failure of enzyme activity. In this report we present the first case in which this type of peroxidase deficiency was associated both with euthyroidism, and with the presence of a non-butanol extractable serum iodoprotein.

Case report. NR, a 22-year-old female, is the propositus of this study. She and her sister, SR, 30-year-old, are the only 2 daughters of a marriage between a euthyroid goitrous woman, MN, 52-year-old, and a normal man, who has a sister with Graves' disease. SR is euthyroid and has had a goiter since early childhood. She has 3 children: 2 normal body and one euthyroid girl, MC, 7-year-old with a small diffuse goiter. Patient NR underwent neck irradiation, for thymus enlargement, at the age of 8 months. Her thyroid has probably received 300 rads. At the time she was seen by us, in our hospital, we found a small diffuse goiter with a very tiny nodule in the right lobe. Clinical laboratory determinations, and an iodine kinetics study were performed (Table 1). Because of the familiar nature of her goiter, the past neck irradiation, and the very small thyroid nodule, we decided to make a total thyroidectomy. A tracer dose of ^{125}I was given, for tissue labelling, 3 days prior to operation. During the iodine kinetic study, an attemp was made to identify the serum non-butanol extractable radioiodine (NBE ^{125}I), which was indistinguishable from iodoalbumin by agar gel electrophoresis. Laboratory and functional thyroid parameters of this family are also shown in Table 1.

Tissue sources. The propositus gland was divided into several pieces immediately after operation. Some of them were examined histologically and a hyperplastic gland with many compact follicles was found. Two other specimens of human thyroid tissue (used as controls) were obtained from patients who underwent thyroidectomies for Graves' disease and multinodular colloid goiter.

Enzymatic studies. Particulate and digitonin pseudosolubilized peroxidase and NADPH-Cytochrome c reductase were prepared by subcellular fractionation. Proroxidase activity was determined by the tyrosine-iodinase and guaiacol assays. NADPH-Cytochrome c reductase activity was also measured.

Iodoprotein studies. Immunoelectrophoresis of thyroid supernatants was performed on agar plates. Polycrylamide-disc-gel electrophoresis of the thyroid supernatants was run in duplicate. One gel was stained with amido-black, while the other was cut in small pieces for counting ^{125}I radioactivity. A correlation between the stained protein bands and radioactive zones was made. Thyroglobulin purification was achieved by salt fractionation and gel filtration on Sephadex G-200. The tissue thyroglobulin concentration was calculated from the specific radioactivity of both the purified protein and the whole thyroid supernatant.

Enzymatic activities. Peroxidase activity was markedly decreased in the congenital goiter (Table 2). The 2 glands, used as controls, had normal activities, towards the lower and the upper limits, respectively. Preincubation of peroxidase preparations with 5×10^{-5} M hematin increased the enzymatic activity of the propositus goiter by 30%, which is commonly observed with normal human

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thyroperoxidases (2). The highest specific activity of the goiter particulate peroxidase preparations was found in the microsomal fraction obtained at 105,000 x g.

Iodoprotein studies. Of the total radioactivity of the whole goiter homogenate, 82.5% was found in the supernatant fraction, 2.5% as free iodide and 80% as iodoprotein. 95% of ^{125}I was found in thyroglobulin by polyacrylamide-gel electrophoresis. The remaining 5% of radioiodide overlapped with the albumin marker.

In this report we present a new example of the deficient peroxidase defect' This congenital error seems to be characterized by: 1) little or no thyroperoxidase activity, 2) normal response of the enzyme to hematin, and 3) normal behavior of peroxidase when analyzed for qualitative properties. Patient NR appears to be the first case in which this defect is associated with euthyroidism and a non-butanol extractable serum iodoprotein, which behaves like iodoalbumin in agar-gel electrophoresis.

1.- Niepomniszcze, H., Castells, S., De Groot, L.J., Refetoff, S., Kim, O.S. Rapoport, B. and Hati, R., J. Clin. Endocr., 36:347, 1973.

2.- Niepomniszcze, H., De Groot, L.J. and Hagen, G.A., J. Clin. Endocr., 34: 607, 1972.

3.- Valenta, L.J., Bode, H., Vickery, A.L., Caulfield, J.B. and Maloof, F., J. Clin. Endocr., 36:830, 1973.

TABLE I

Laboratory, functional, and iodine kinetic parameters of the goitrous family

Test	Units	Patients				Normals
		NR	SR	MM	MC	
Total serum T4 (by C.P.B.)	µg/100 ml	5.5	7.6	7.9	8.4	5.5-13.7
Total serum T3 (br R.I.A.)	ng/100 ml	80	-	-	-	75-210
Serum TSH by R.I.A.). . . .	µU/mi	69	0.9	10.8	0.7	<1-8
Antihyroglobulin antibodies (by TCR).	titer	negat.	negat.	negat.	negat.	<1/30
RAIU-24-hr (basal conditions)	%	19	35	30	18	20-40
Perchlorate discharge at 60 min (basal conditions). . . .	%	posit. 65	posit. 9.5	negat.	negat.	negative 0-9
Perchlorate discharge at 60 min (after TSH stimulation) .	%	-	posit. 43	negat.	negat.	negative 0-9
RAIU-24-hr (beginning 24 hr after administration of $KClO_4$).	% of basal	6	39	-	-	16-70
RAIU-24-hr (beginning 48 hr after administration of $KClO_2$).	% of basal	34	110	-	-	63-101
RAIU-24-hr (beginning 9 days after administration of $KClO_4$).	% of	106	-	-	-	100
PBI-125 (48 hr)	%/1	0.6	0.1	-	-	up to 0.2
NBEL-125 as percent of PBI-125	%	2.8	34	-	-	0
In vivo dehalogenation test of MIT-^{131}I	% of dehalogen.	100	-	-	-	100
Thyroid iodide elearance (C_G)	ml/min	57.7	-	-	-	16
Thyroid I uptake (AIU)	µg/d	80	-	-	-	120
Thyroid iodine release (H)	µg/d	54	-	-	-	120
Thyroidal iodide leakage	µg/d	26	-	-	-	1.2
Plasma inorganic iodide (PII)	µg/d	1.77	-	-	-	
Thyroid iodine release slope (K_G#2).	day/1	0.277	-	-	-	0.012
^{127}I content of 24 hr urine colection (E)	µg	148	-	-	-	198
Total thyroid iodine (kinetic analysis) Q_G	µg	156	-	-	-	15.000
Total thyroid iodine (chemical)	µg	600	-	-	-	
Biological half life of serum T4	days	2.9	-	-	-	6.5
Biological half life of serum T3	days	0.75	-	-	-	1.5
Daily degradation of iodine-T4	µg	48	-	-	-	57.7
Daily degradation of iodine-T3	µg	5.94	-	-	-	19.0
Extrathyroidal body T4 pool	µg of I	188.3	-	-	-	483.3
Extrathyroidal body T3 pool	µg of I	6.43	-	-	-	41.0

TABLE 2

Thyroid peroxidase activity

Specimen	Tyrosine-iodinase assay* (using particulate enzyme)	Guaiacol assay** (using digitonin pseudo-solubilized enzyme)
Familial goiter (fresh tissue)	45	
Familial goiter (frozen tissue)	65	61
Multinodular goiter (as control)	214	177
Graves' disease (as control)	410	260
Normals (Niepomniszcze et al. 1975)	220-410	

* Activity is expressed as nmol of I incorporated into tyrosine/g of tissue.

** Activity was measured by spectrophotometry, and expressed in Units. One Unit is equal to Δ O.D. (470 nm)/10 sec/mg of protein x 1000.

C. 5 Effect of Gamma Radiation on the Antibody Molecule (Ab).

I. Immnulogical Studies of Anti Human Thyroglobuline (Rabbit Serum)

L.M. Scavini, S.M. Rodriguez, J.O. Degrossi* and E. Mariano*.

In previous studies experiments on the modifications produced in antigenic determinants of human immunoglobulin was carried. These modifications were results of gamma radiation effect on Fab and Fc fragments. After these results we decided to investigate the effect of gamma radiation on the Ab combining site. With purified human thyroglobulin a specific immune serum was obtained in rabbits.

Lyofilized samples in solution (10 mg/ml) were irradiated with an external gamma-ray source. The dose varied between 0.5 and 15.0 Mrads.

Antibody titers were measured by passive hemagglutination with formolinized sheep erythrocytes sensitized with the antigens.

With the samples in solution the following Ab concentration in order to obtains positive results were necessary: 0.02 µg AbN/ml for 0.5 Mrad, 0.15 µg AbN/ml for 1 Mrads; 1.20 µg AbN/ml for 2 Mrads; 2.5 µg AbN/ml to 3 Mradas; 20 µg AbN/ml for 4.0 Mrad and 40 µg AbN/ml for 5 Mrads.

With the lyofilized samples it was observed that up to 3 Mrads the required concentration of Ab did not vary. After this dose the required concentracion increased in the following way 0.02 µg AbN/ml for Mrads, 0.15 µg AbN/ml for 10 Mrads, 2.50 µg AbN/ml µg AbN6ml and for 10 Mrads 10 µg AbN/ml.

This data suggest a dose of 3 Mrads of gamma rays for preservation of immune serum against human thyroglobulin.

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* Nuclear Medicine Center, Buenos Aires.

C. 6 Effect of Gamma Radiation on the Antibody Molecule.

II. Immunological Studies of Anti-Human Serum Albumin (HSA) (Rabbit-Serum).

S.M. Rodriguez, L.M. Scavini and E.E. Capalbo.

Anti-human albumin serum was obtained by immunizing rabbits with saline precipitation and D E A E Cellulose purified HSA.

Lyofilized samples were exposed to gamma radiation of an external Co 60 source using doses of 0.5 to 20.0 Mrad.

Passive hemaglutination and quantitative precipitation analysis were the techniques used to investigate the modification of the combining sites of Ab with the antigenic determinants of HSA-I-^{125} (specific activity of 100 µCi/mg).

With these experiments it was observed that by passive hemagglutination it was necessary to have 0.02 µg NAb/ml to give a possitive reaction when the gamma ray dose was 1.0 Mrads. For 3.0 Mrad, 0.08 µg Nab/ml; for 5.0 Mrad, 0.15 µg NAb/ml; for 10.0 Mrad 0.64 µg NAb/ml; for 15.0 Mrad, 1.20 µg NAb/ml and for 0.20 Mrad, 2.50 µg NAb/ml the affinity with the Ag (1.60 µg AbN/ml) decreased 55% for doses of 1.0 to 20.0 Mrad.

Beyond 10.0 Mrad the combining site with the Ag was destroyed. It is concluded that lyofilized anti HSA antibody exposed to doses smaller than 3.0 Mrad show a high affinity.

D. MICROBIOLOGY

D. 1 Isolation of Envelope Mutants in Salmonella typhimurium.

D.N. Antón.

The action of radiation on DNA replication and cell division is well known. Taking into account that these two functions appear to be controlled by the cell envelope, a research program aimed to investigate the role of the envelope in the response of cells to radiation has been started. This program comprises the isolation and physiological and genetic characterization of envelope mutants in Salmonella typhimurium, and the study of effects produced by those envelope alterations in the normal response of cells to radiation.

A system that allows positive selection of mutants carrying envelope defects has been found in S. typhimurium. Strain DA78 behaves as a very poor recipient for the mutation his01242, and very few transductants arise when this histidine regulatory mutation is transduced into DA78. Microscopic studies have shown that the introduction of his01242 in DA78 cells produces striking changes in their morphology. Long, frequently forked, filaments carrying in their middle part a very large balloon, are formed. Those ballons break eventually causing cell death (1).

A few DA78 cells are able to withstand the action of his01242 without losing entirely their capacity to grow. Thus, those cells are positively selected since they are the only producers of his01242 transductants. Most of the transductants formed grow poorly and show genetic instability. They segregate colonies that differ from the original strain in colonial morphology and growth capacity. By successive reisolations of 12 his01242 transductants from strain DA78, 41 colonies displaying improved growth and modified appearance were obtained. As shown in Table 1, many alterations in functions related to the envelope appear among those strains. In several cases multiple defects were carried by the same strain.

The properties of the 11 strains displaying cell shape anomalies were studied. Nine of them make spherical instead of elongated cells, and the other two show atypical cell shape although no round cells are made. At least three genes concerned with cell shape are represented in this group of mutants. The alteration present in strain DA82 is cotransducible with cod (min. 105) whereas the one carried by strain DA150 is jointly transduced with hisS (min. 78) (2) and belongs, probably, to a cell shape gene already described in Salmonella (3). The location of the remaining mutations is still unknown, yet, it has been shown that at least six of them are not cotransducible with either cod or hisS markers. The cell shape of the mutants in the last class is rather irregular and appears to be influenced by the nutritional composition of the growth medium.

Autolysis of the 41 strains and corresponding control strains was tested in Tris-HCl buffer, 0.05 M, pH 8.5 at 37°. Three autolytic levels were found: 1) normal, shown by 32 strains, produces a 20% decrease in the optical density (OD) of the cell suspension after 2 hours of incubation; 2) intermediate, shown by three strains, produces OD decreases of 30%; and 3) high, shown by six strains, produces a 40 % of lysis.

The strains displaying high autolysis can be further divided into two types. One, composed by envD mutants (DA126, DA149, and DA123, which appears to be a point envD mutant) shows lysis when broth becomes alkaline, and is unable to growth in nutrient agar pH 9.5 at 43° (4). The second class is formed by strains DA130, DA138 and, probably, DA82; they do not lyse spontaneously in

broth and grow normally in alkaline agar, yet, they reach in buffer the same autolytic level as the other three strains.

The strains displaying an intermediate level of lysis show also normal growth and uncover their autolytic power only when placed in buffer. The three strains in this class make round cells; so they also carry a morphological mutation. By analyzing for cell shape and autolysis hisS$^+$ transductants obtained with phage grown on DA150, it has been possible to demonstrate that in strain DA150 the same mutation that produces the alteration in cell shape causes the increase in autolysis.

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2.- Palermo, A.M., personal communication.

3.- Wyche, J., Kennedy, J., Hartman, Z., Hartman, P. and Diven, J., J. Bacteriol., 120:965, 1974.

4.- Antôn, D.N. and Orce, L.V., Molec. gen. Genet., 144:97, 1976.

TABLE 1

Alterations found in 41 derivatives his01242 from strain DA78

Alteration	Number of strains carrying it
Abnormal cell shape	11
Sensitivity to deoxycholate (3 mg/ml)	9
Sensitivity to cycloserine (20 µg/ml)	7
Autolysis	6
Sensitivity to novobiocin (75 µg/ml)	5
Sensitivity to acridine orange (100 µg/ml)	5
Thermosensitivity (43°)	3
Sensitivity to EDTA (5 x 10^{-4} M)	2
Sensitivity to UV	1
Osmotic sensitivity	1
Apparently normal	17

D. 2 Autolytic Activity of a Mutant of Salmonella typhimurium.

L.V. Orce and D.N. Antón.

The autolytic activity of bacteria has been known for a long time; however, the role of the autolytic enzymes is not clear. They have been implicated in the process of septation, in cell elongation, and as "dechaining" enzymes. An envelope mutant has been isolated in Salmonella typhimurium that lyses spontaneously in nutrient broth. The present report depicts the autolytic behavior of strain DA126.

Strain DA126 grows normally in minimal medium and nutrient broth; yet, it suffers a steady autolysis 2-3 hours after reaching post-exponential phase in the latter medium. The phenomenon does not occur when nutrient broth is supplemented with Mg^{2+} or glucose (Fig. 1).

Autolysis seems to be independent of macromolecular synthesis. Interference with the synthesis of protein by tetracycline or chloramphenicol, RNA by 5-fluorouracil, and DNA by nalidixic acid or phenethyl alcohol does not affect the autolytic performance. The synthesis of protein, RNA, and DNA, followed by labeling the cells with ^{14}C-L-leucine, ^{14}C-uracil, and ^{3}H-thymidine respectively, did not show differences from the control. The degradation of those macromolecules was also normal.

The effect of glucose in preventing lysis does not seem to be related to energy requirements. Neither respiration inhibitors (Na azide) nor oxidative phosphorylation inhibitors (oligomycin or rutamycin) and uncouplers (2.4-dinitrophenol or arsenate) were able to neutralize the protective action of glucose. Furthermore, they did not prevent lysis in broth cultures without the sugar.

Glucose protection appears to be related to pH changes. Except for Mg^{2+}, whose activity does not appear to be connected with pH, protection by other agents correlated well with the pH of the medium after 24 hours. According to the results obtained the autolytic activity of DA126 needs alkaline conditions to be displayed.

Lysis take place much faster when cells are resuspended in appropiated buffer solutions. By incubating DA126 cells in buffer at several pH's it was found that lysis started immediately and was faster with increasing pH (Fig. 2). When the rate of lysis (K) was plotted against pH a direct relationship was found up to 10.5 the highest pH tested (Fig. 2, inset). Lysis depends also on buffer ionic strength and incubation temperature. As shown in Fig. 3, a linear response between rate of lysis and temperature was found in the range from 25 to 45°C. An activation energy (Ea) of 13.4 kcal per mole was calculated from the slope of that line. The Ea varies with pH, and shows the lowest value between pH's 8.5 and 9.5 (Fig. 3, inset).

The inhibition of lysis at low pH was not permanent, and the same was true for Mg^{2+} protection. On the contrary, lytic activity was completely lost when cells were boiled for 5 min., or treated with $HgCl_2$ or AgCl for 30 min. at 0°C. Yet, similar treatments with other sulphydryl reagents such as N-ethylmaleimide, iodoacetamide, or p-chloromercuribenzoate proved ineffective.

Partially purified cell walls showed in buffer the same lytic pattern as whole cells. When merein, extracted from DA126 and the wild type LT2, was submitted to the action of increasing pH's, the same results were obtained with either DA126 or LT2 mureins, suggesting that murein per se is not responsible for DA126 lytic behavior.

The appearance of reactive groups in the supernatant of lysing walls was studied in order to obtain information on the type of autolysin involved. There

was a progressive increase in amino terminal groups while reducing groups remained constant. Thus, these results point to an amidase or an endopeptidase as the lytic enzyme. To identify the aminoacid responsible for the increase in N-terminal groups, cell walls were allowed to lyse and samples of the supernatant were treated with 2.4-dinitrofluorobenzene (DNF). DNF-aminoacids were separated by thin layer chromatography and compared with samples of pure DNF-aminoacids. This procedure allowed the identification of diamino pimelic acid as the aminoacid providing the N-terminal groups in the supernatant of DA126 lysates. This result suggests that the autolytic enzyme is a D-alanyl-(D)-meso-diamino-pimelyl endopeptidase.

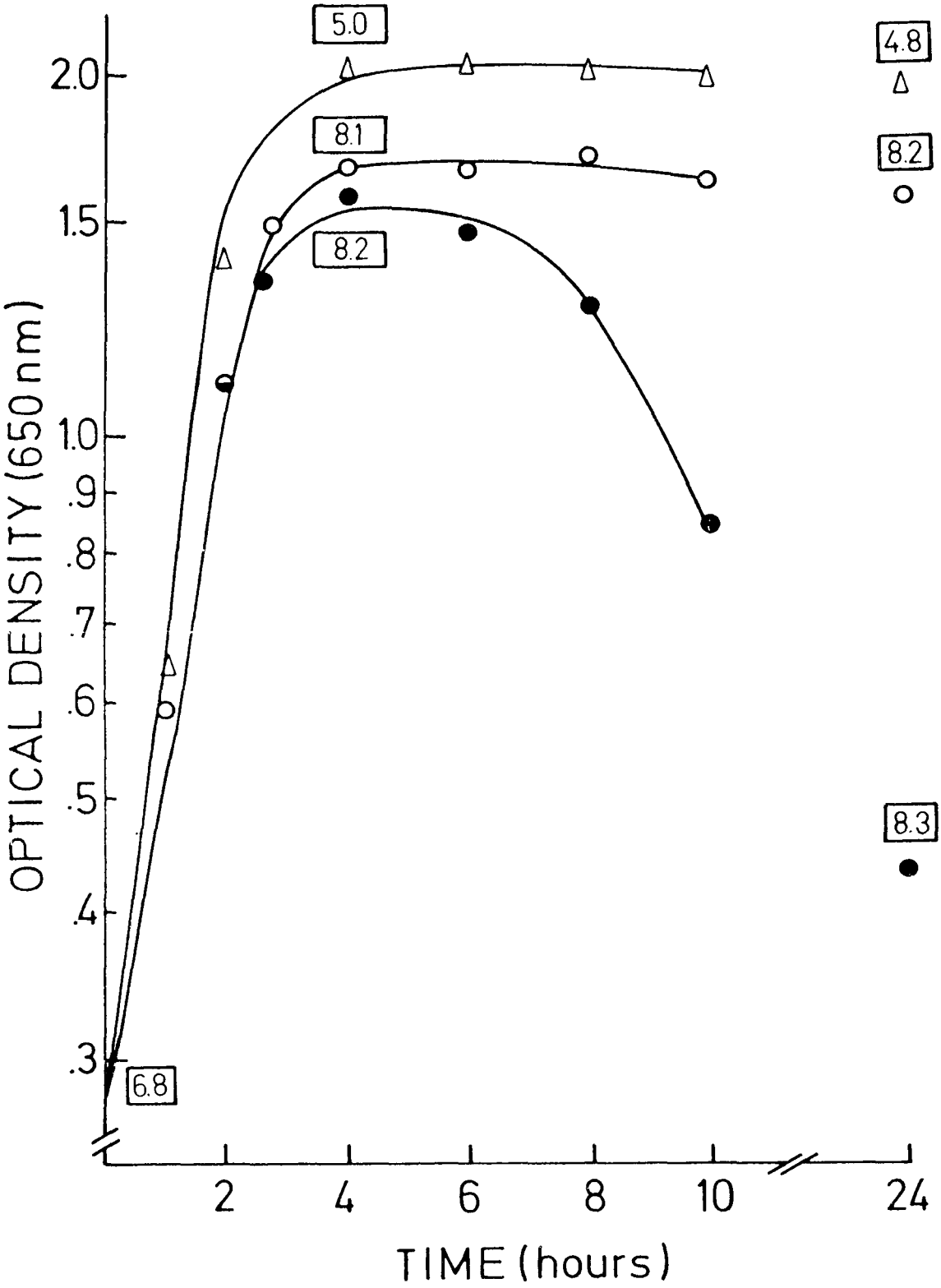

Figure 1.- Growth curves of S. typhimurium DA126 in nutrient broth (●) and same supplemented with 0.05 M $MgCl_2$ (○), or 0.05 M glucose (△). Numbers in boxes represent pH values.

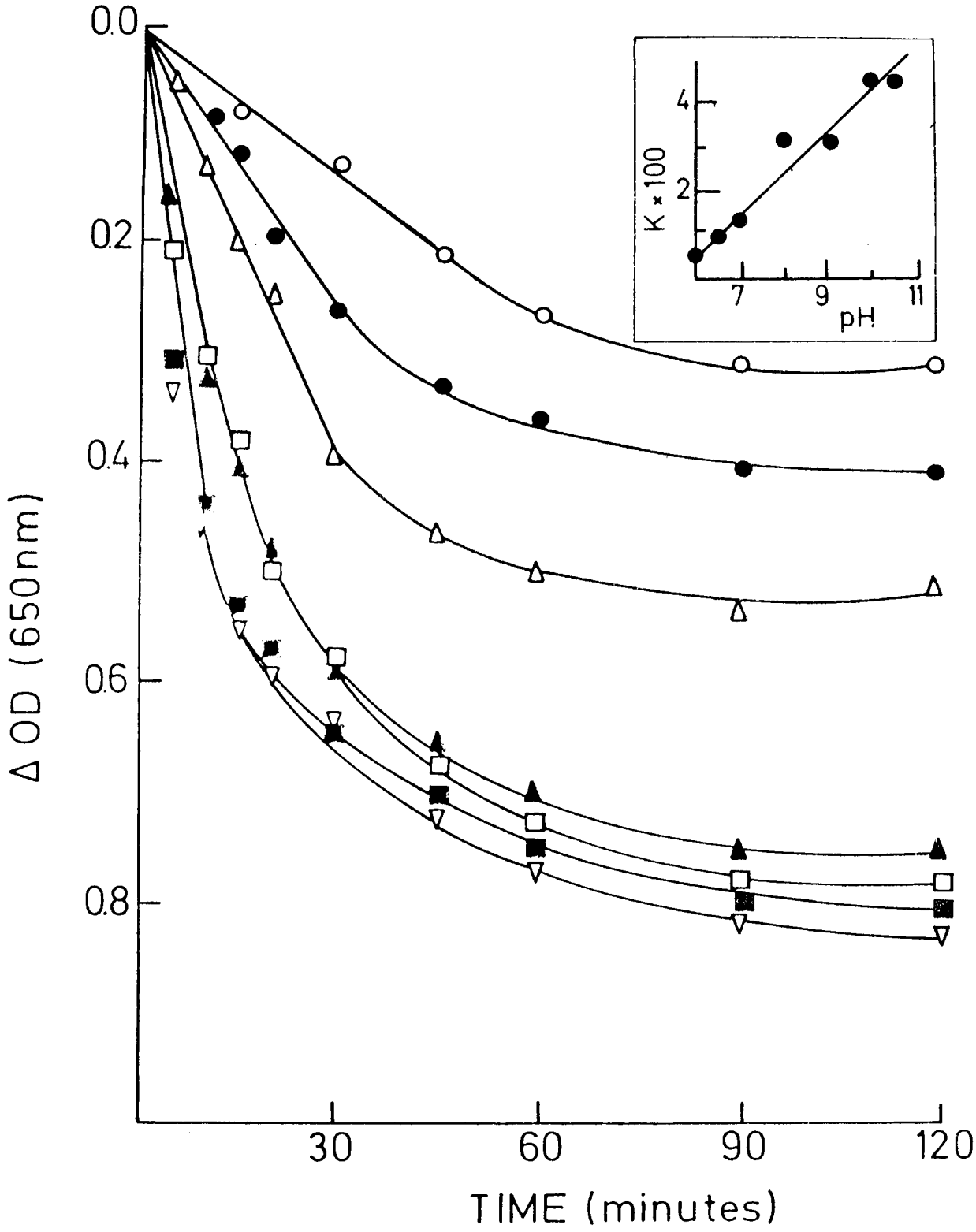

Figure 2.- Effect of pH on the rate of lysis of DA126 at 37°C. The inset shows rate of lysis (K values) as a function of pH: pH 6.0 (○); pH 6.5 (●); pH 7.0 (△); pH 8.0 (▲); pH 9.0 (□); pH 10.0 (■); pH 10.5 (▽).

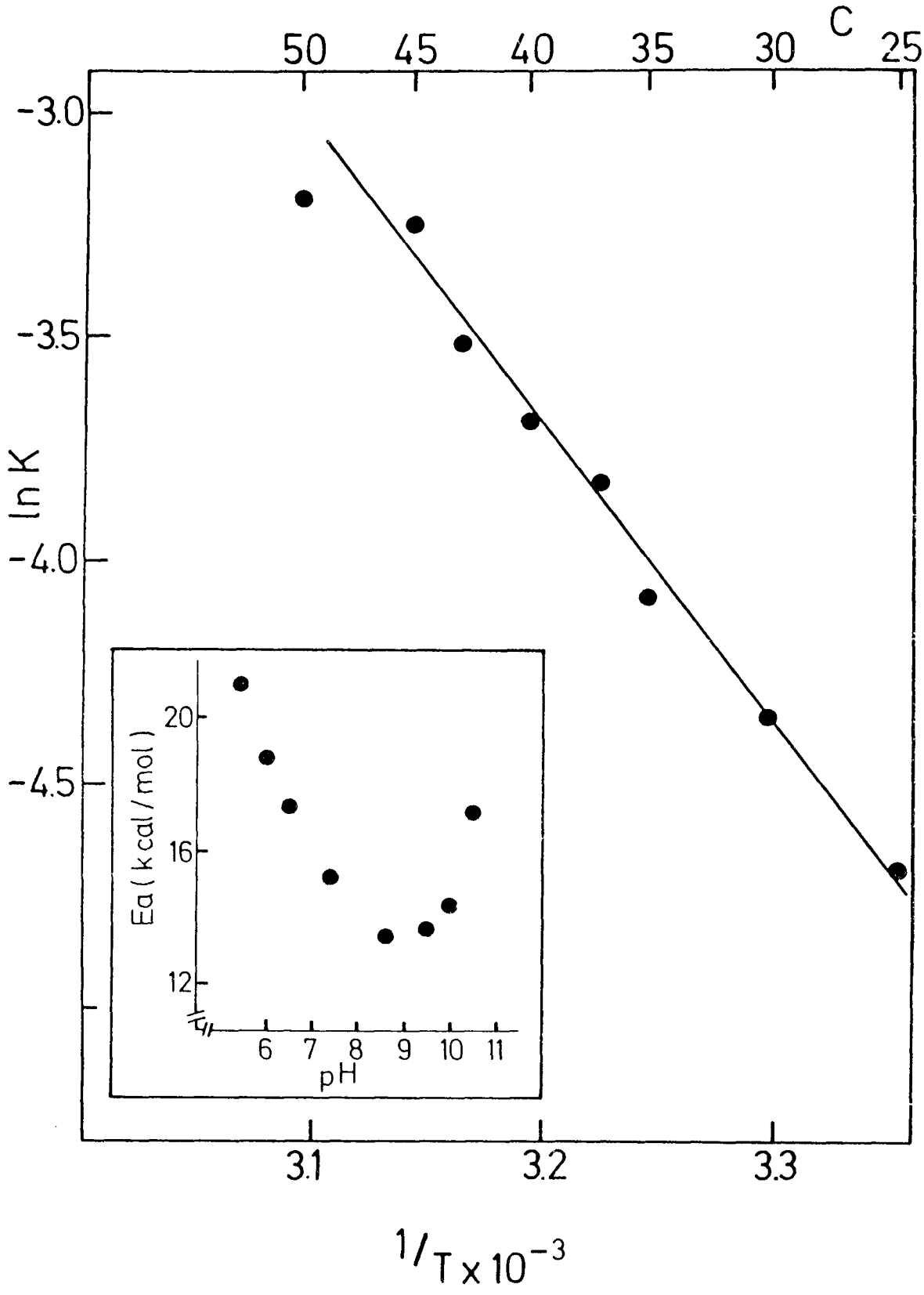

Figure 3.- Effect of temperature on autolysis at pH 8.6. The inset shows the energy of activation (Ea) as a function of pH.

D. 3 Envelope Mutation Promoting Autolysis in Salmonella typhimurium

D.N. Antón and L.V. Orce

Involvement of autolytic enzymes in cell growth and division has been frequently assumed even though direct evidence is scanty. Among the envelope mutants isolated in S. typhimurium two strains displayed growth characteristics that suggested the presence of abnormal autolytic activity (1). These two strains, DA126 and DA149, were selected for further study since they could provide evidences on the role of autolysins in normal growth and division, and help to understand the inhibitory action of radiation on cell division.

Both mutants grow normally in minimal medium but in nutrient broth low turbidities are observed in overnight cultures. This is not due to impaired growth but to the slow exponential lysis that develops after stationary phase is attained. In addition, both strains show increased sensitivity to deoxycholate (DOC, 2 mg/ml), sodium dodecyl sulfate (SDS, 0.8%), and EDTA (1.0×10^{-3} M).

The autolytic mutations carried by DA126 and DA149 are quite stable since no revertants capable of growing on alkaline nutrient agar (ANA) at 43° could be found either spontaneously or after treatment with nitrosoguanidine and diethylsulfate. The locus involved in autolysis has been named envD, to differenciate it from other envelope loci already identified in the related organism Escherichia coli.

To locate the envD locus crosses among $envD^+$ Hfr strains and auxotrophic derivatives of DA126 were performed. The results suggested that the pertinent locus is located in the sector from 20 to 50 min. in the conventional linkage map of Salmonella. In transduction tests, mediated by phages P22 and KB1, envD was found to be jointly transduced with the gal marker. Similar cotransduction frequencies were found for the autolytic mutation from DA126 (16,8%) and that from DA149 (27.8%). These results indicate that both mutations belong to the same or very close loci. Furthermore, no recombination between them could be detected. Several $envD^+$ transductants of strain DA126 were tested for response to DOC, EDTA, and SDS. All of them had recovered normal resistance, thus indicating that autolysis and drug sensitivity are caused by the same mutation.

In order to obtain a more precise location of envD, a strain carrying mutations in genes nadA and aroG, both closely linked to gal, was constructed. Phage grown on this strain was used to transduce a gal derivative of strain DA126; $envD^+$ transductants were selected and analyzed for unselected markers. The results, shown in Table 1, indicate that envD is located counterclockwise from nadA and very close to it since they showed more than 50% joint transduction. This data, together with results reported for sucA (2), indicate that envD is located between genes sucA and nadA (Fig. 1).

In order to make sure that P22 prophage, carried by both strains, does not contribute to autolysis, an attempt to obtain a non-lysogenic envD strain was made. The procedure employed involved several successive crosses and, finally, a transduction that yielded a pair of non-lysogenic P22-sensitive strains differing only in envD allele (DA361, envD1; DA362, $envD^+$). Analysis of these isogenic strains confirmed that the autolytic behavior of envD strains is not connected with the presence of P22 prophage.

envD also affects morphological characteristics of the cells. Using phase contrast microscopy it was observed that a significant proportion of DA126 cells are shorter than wild type cells. Furthermore, they appear frequently associated in short chains of 4 to 10 cells. Strain DA149 also makes cell chains, but the

frequency of short cells is lower than in DA126 cultures. Introduction of the *envD*$^+$ allele by transduction allows recovery of the normal appearance.

Electron microscopy of DA126 and DA149 cells allowed another abnormality of *envD* mutants to be detected. Murray and co-workers (3) demonstrated that cell division by constriction, as shown by wild type cells under standard conditions, is an artifact produced by fixation. Yet, even under standard conditions, septation was the only type of division detected in *envD* mutants.

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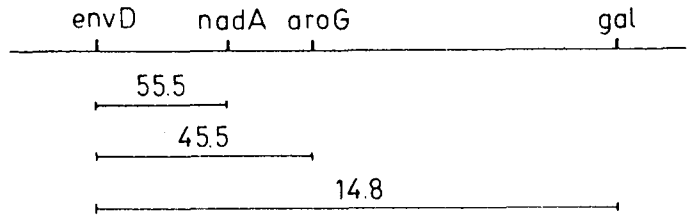

Figure 1.- Gene order and frequencies of joint transduction (%) according to the data shown in Table 1.

TABLE 1

Genetic analysis of 189 *envD*$^+$ recombinants selected in a transduction between DA236 (*gal envD1*) and phage grown on DA247 (*nadA13 aroG*)

Recombinants classes obtained			N° of colonies in class	N° of crossovers required according to the gene order shown in Fig. 1
nadA	aroG	gal		
+	+	-	81	2
-	-	-	58	2
-	-	+	26	2
-	+	-	21	2
+	-	-	1	4
+	+	+	1	4
+	-	+	1	4
-	+	+	0	4

D. 4 Genetic Analysis of an Osmotic-Sensitive Mutant of *Salmonella typhimurium*

D. N. Antón.

It has been found that one of the strains isolated in *Salmonella typhimurium* displays several alterations in functions related to the envelope. It makes round instead of elongated cells as the wild type does. Moreover, the abnormal osmotic properties of the strain produce exponential inactivation when the cells are transferred from a medium with normal osmolality to another having low salt content. These alterations are accompanied by increased sensitivity to antibiotics that interfere with the synthesis of murein, such as penicillin and cycloserine (1).

The genetic study of strain DA82 was started in order to: a) find out whether each of the alterations observed in DA82 is caused by an independent mutation, b) locate all the mutations present in the strain, and c) construct pairs of strains isogenic for all but the mutation under study.

Several normal strains belonging to different Hfr types were crossed with auxotrophic mutants isolated in strain DA82. The genetic analysis of the recombinants obtained in those crosses located the gene controlling cell shape and osmotic sensitivity (*bac*) near *strA* (minute 108) in the genetic map of *Salmonella*. Donor strains carrying the *bac*$^-$ allele were constructed by crossing F$^+$ strains with appropiate DA82 derivatives. The *bac*$^-$ donors obtained were crossed with recipients carrying mutations in genes located near *strA*; in this way the place for gene *bac* was limited to the segment 102-108 min., between markers *argE* and *strA*. The same crosses suggested strongly the location for *bac* to be between *cod* and *strA* (min. 104-108).

Taking into account the small size of the region involved and the availability of convenient markers, the probable cotransduction of *bac* with close markers was investigated. Numerous tests employing the usual *Salmonella* phages P22 and KB1 were carried out with negative results (Table 1). The failure to demonstrate cotransduction between *bac* and neighboring markers would imply that the distance between them is larger than the DNA fragment transduced by the phages employed (approximately 2.6×10^7 daltons). No transducing phage able to carry larger pieces of DNA is known for *S. typhimurium*; on the other hand, it has been shown that *Escherichia coli* phage P1 transduces fragments of about 6×10^7 daltons. Recent studies have demonstrated that the resistance of *S. typhimurium* to phage P1 is due to lack of adsorption, and strains sensitive to P1 were identified among mutants carrying specific alterations in licopolysaccharide synthesis (2). Phage P1 grows quite normally on the sensitive mutants and its behavior in transduction is very similar to that shown in *E. coli* strains (3).

To check joint transduction of *bac* by phage P1, mutants sensitive to the phage were isolated in several strains carrying either *bac* or other convenient markers. The corresponding crosses were performed and joint transduction of *bac* with genes *argE*, *cod*, *aroC*, and *strA* was detected. The frequencies of cotransduction observed (Table 1) allow the construction of the map in Fig. 1, where the physical distances depicted have been calculated according to Kemper's equation (4).

Pairs of isogenic strains differing only in *bac* allele were obtained in the transductions mediated by phage P1. Preliminary studies of these strains indicate that the mutation *bac* is widely pleiotropic since it affects not only cellular shape and osmotic behavior but also sensitivity to penicilin and cycloserine. Furthermore, it confers increased sensitivity to deoxycholate (3 mg/ml) and novobiocin (75 µg/ml), and alters the utilization of several carbohydrates.

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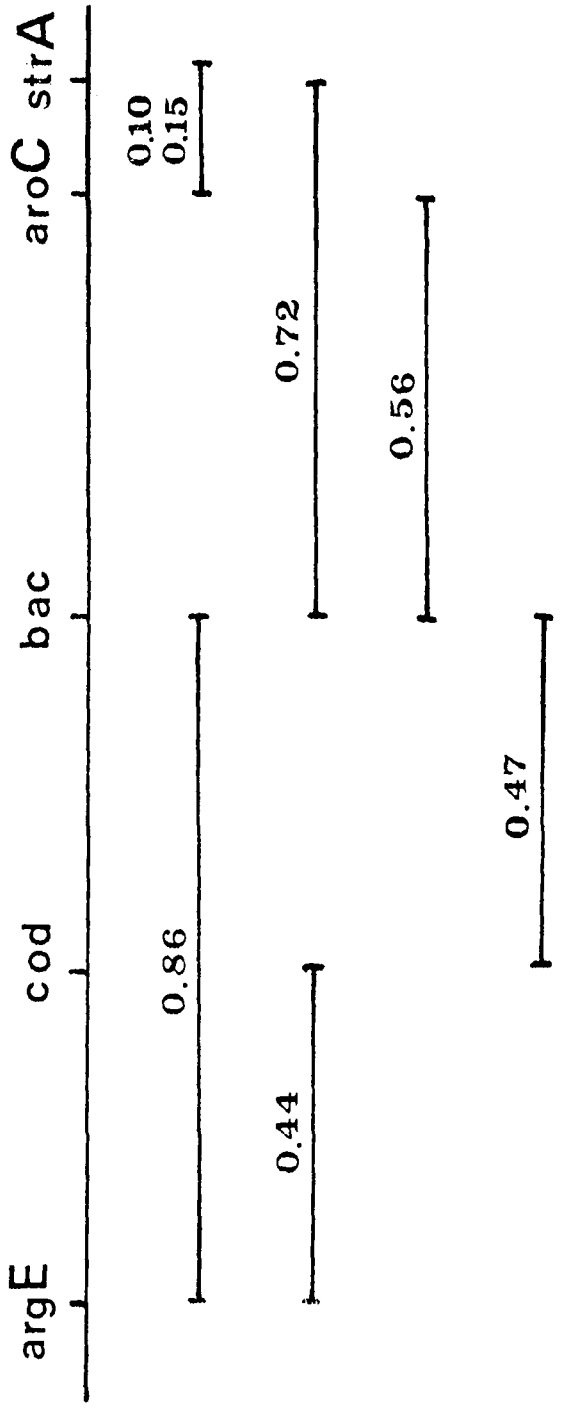

Figure 1.- Gene order and physical distances (calculated as described by Kemper (4)) between bac and neighboring markers.

TABLE 1

Between markers	Frequency of cotransduction (%) with phage	
	KB1	P1
argE*-bac	<0.5	1.0
argE*-cod	<0.5	20.0
cod*-bac	<0.2	17.4
aroC*-bac	<0.5	11.7
aroC*-strA	4.5	56.6
strA*-aroC	-	67.2
strA*-bac	-	4.3
aroC*-strA-bac	-	1.8
bac*-aroC	<0.5	-

* Selected marker.

D. 5 Effects of Osmotic Downshock on Macromolecular Synthesis in an Osmotic-Sensitive Mutant of Salmonella typhimurium

D.N. Antón and C.J. Quintans.

Previous work has shown that when the osmotic-sensitive mutant DA82 is submitted to osmotic downshock (O.DS.) in nutritive medium, growth and cell division stop and exponential cell death occurs (1). In this report results on the effects of O.DS. on macromolecular synthesis in DA82 are presented.

The behavior of DA82 after transfer from normal to downshock medium depends on the nature of both media. It was found that DA82 cells showed a much higher survival when transferred to downshock minimal medium (E-20) than when shifted to a rich medium of similar osmolality (NoS-broth). On the contrary, cells grown in minimal medium (E) were more sensitive to osmotic downshock than cells grown in S-broth). Therefore, the strongest effect was observed when E-grown cells were put into NoS-broth. In some experiments cells grown in E medium were transferred to E-20 medium. This treatment produced inhibition of growth and cell division but no significant death. After a lag of approximately 4 hours cells recovered and restarted growth.

As shown in Fig. 1A, transfer of E-grown cells to NoS-broth immediately halted the incorporation of labeled uracil and leucine into cold TCA insoluble material. Incorporation of thymidine, though strongly depressed by O.DS., kept going for at least 200 minutes. Addition of NaCl to downshocked cultures resulted in the rapid resumption of growth and cell division. Similarly, incorporation of thymidine, uracil, and leucine started again (Fig. 1A).

The same pattern was found when synthesis of DNA, RNA, and protein was followed after O.DS. in E-20 (Fig. 1B). Yet, in this case, downshock produced only a very slight drop in viability. Hence, osmotic death would not be caused by the inhibition of macromolecular synthesis produced by D.OS.

Degradation of macromolecules in cultures of DA82 submitted to downshock in either NoS-broth or E-20 medium was followed. No significant loss of the radioactive precursors incorporated previously into DNA (^{3}H-thymidine), RNA (^{14}C-uracil), and protein (^{3}H-leucine) was observed in cells kept in downshock medium for as long as 5 hours. Thus, inhibition of synthesis is not accompanied by increased breakdown of the macromolecules.

In order to see whether inhibition of RNA synthesis is limited to stable species or affects also mRNA, induction of β-galactosidase was followed during downshock. Since Salmonella does not utilize lactose an F'lac^{+} factor from E. coli K12 was transferred to a DA82 derivative. The strain was grown in E medium and submitted to downshock in NoS-broth. Samples from NoS-broth and S-broth cultures were withdrawn at intervals and treated with IPTG to induce synthesis of β-galactosidase mRNA, or with water for uninduced control cultures. The results indicate that osmotic inhibition does not prevent the synthesis of mRNA since a considerable amount of β-galactosidase activity was measured in the induced NoS-broth samples.

As the behavior of DA82 after O.DS. resembles the stringent control response displayed by normal cells after starvation for amino acids, the synthesis of PPGPP and PPPGPP in downshocked cultures was studied. It is well known (2) that the stringent response is characterized by the accumulation of those two nucleotides in the cells. The results obtained with DA82 demonstrate that the effects of O.DS. on macromolecular synthesis do not involve the stringent mechanism since no increase in the level of PPGPP and PPPGPP during downshock was observed.

It is suggested that normal macromolecular synthesis is dependent on a particular membrane conformation, and that the effects of osmotic downshock are due to the excesive stretching of the membrane allowed by DA82 defective cell wall.

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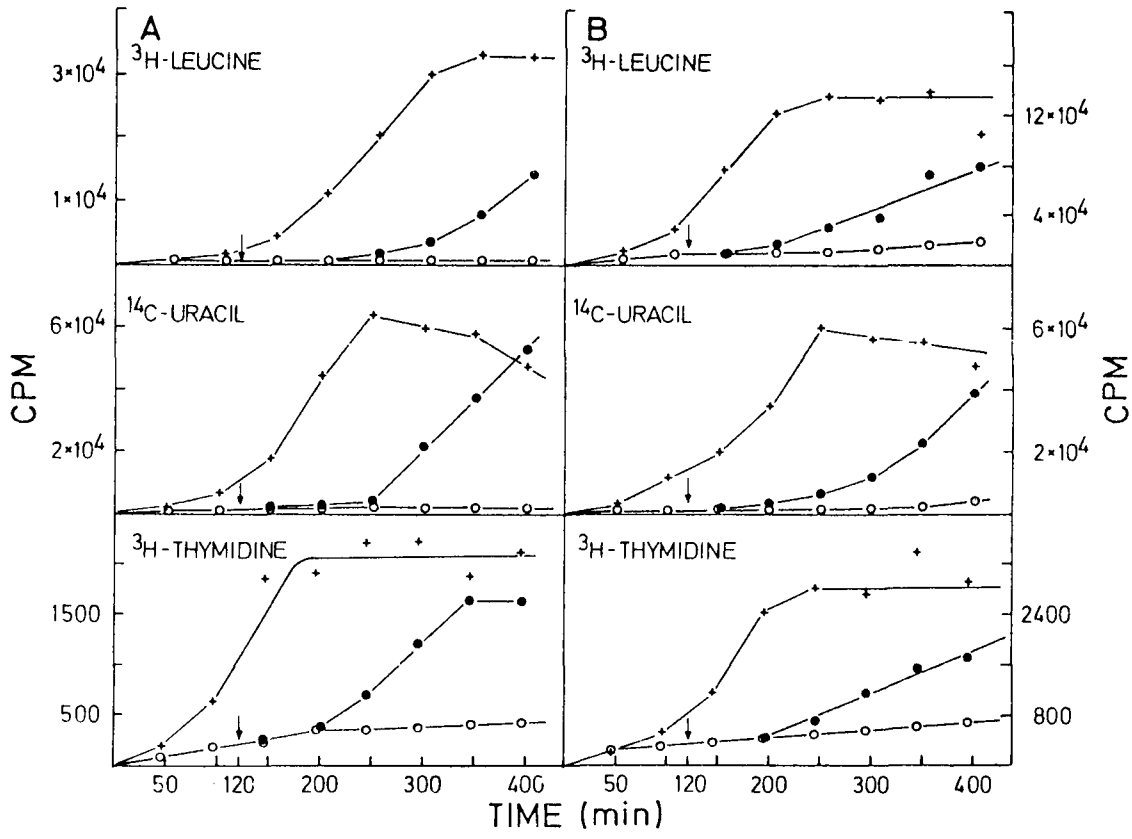

Figure 1.- Effects of osmotic downshock on the incorporation of labeled precursors into DNA, RNA, and protein. A: cells grown in E medium, transferred to NoS- (o) and S-broth (+) at 0 time. B: cells grown in E medium,transferred to E-20 (o) and E medium (+) at 0 time. After 120 min. (arrow), NaCl 0.1 molal was added to portions of NoS-broth and E-20 cultures (•).

D. 6 Changes in Resistance to Ultraviolet Radiation in an Osmotic Sensitive Mutant of Salmonella typhimurium

C.J. Quintans

The influence of medium osmolality on the response of Salmonella typhimurium DA82 (1) to ultraviolet light (254 nm) was studied.

It was found that the osmolality of the medium at the moment of irradiation does not affect the UV. sensitivity of this strain since cells in logarithmic stage of growth displayed the same UV. sensitivity whether they were suspended in minimal medium M9 (2) diluted to 20% (NoS medium) or in the same medium plus 0.1 molal Na Cl (S medium) (Fig. 1). Yet, when previously to the irradiation the cells were incubated in those media, differences in behaviour arose. Cells incubated for 60 minutes in NoS medium suffered a considerable increase in resistance to UV. whereas cells maintained for the same time in S medium showed no change (Fig. 1) The new resistance level of the cultures in NoS medium remained constant for at least five hours of incubation. On the contrary, the UV. sensitivity of the cells incubated in S medium diminished when the culture reached the stationary stage, and only then the resistance level rose to similar levels as those attained by NoS cells several hours before.

Nalidixic acid (15 µg/ml), an inhibitor of DNA synthesis, prevented the development of resistance in bacteria incubated in NoS but was unable to affect the sensitivity of cells incubated in S or to modify the response to UV. light of cells incubated in NoS once the resistance has appeared (Fig. 2).

Chloramphenicol (150 µg/ml) added to NoS cultures was unable to avoid the increase in resistance. However when this inhibitor of protein synthesis was added to S cells, resistance to UV. rose, after one hour of incubation, reaching the same level as that of bacteria in NoS (Fig. 3). When nalidixic acid was added to S cells simultaneously with chloramphenicol, the resistance increase was not observed.

Resistance developed by DA82 after incubation in low osmolality medium resembles in many ways that displayed by cells incubated in normal osmolality medium when they reach the stationary phase or are submitted to treatments which arrest initiation of new replication rounds. Nalidixic acid, which inhibits DNA elongation, prevents resistance changes but is unable to revert it once resistance has appeared. The inhibition of initiation of DNA replication by chloramphenicol produces in S cells and increase in UV. resistance which is concealed (in cells incubated in NoS) by the one caused by the osmolality of the medium.

Resistance to UV. radiation is known to be higher in bacteria with resting chromosomes than in growing cells. It has been postulated that increased resistance could be due to a more efficient repair of UV. radiation damage in cells with complete chromosomes or to a special radiation sensitivity of the replication point (3).

The results presented here suggest that in NoS medium, probably as a consequence of the inhibition of macromolecular synthesis caused by low osmolality (4), DA82 fails to initiate new rounds of DNA replication, becoming, after completion of rounds already started, more resistant to UV. light.

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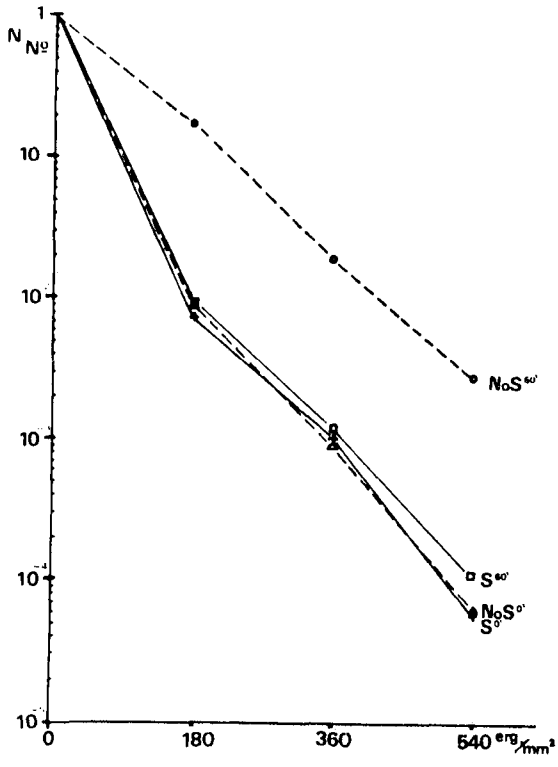

Figure 1.-
Sensitivity of DA82 to UV. radiation.
+——+——+ Suspended in S
Δ- -Δ- -Δ Suspended in NoS
□——□——□ Incubated 60' in S
○- -○- -○ Incubated 60' in NoS

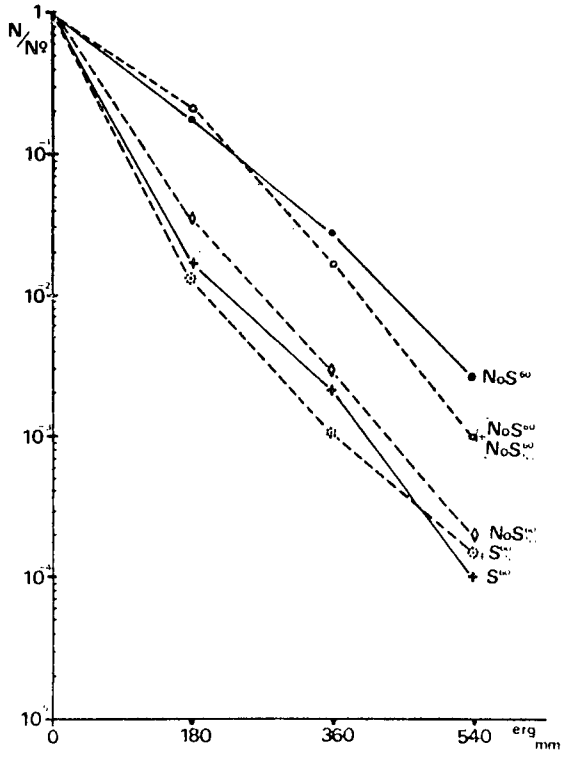

Figure 2.- Effect of Nalidixic acid (15 µg/ml) on the UV. sensitivity of DA82

- Incubated 60' in NoS
- Incubated 60' in NoS plus Nalidixic acid
- Incubated 60' in NoS and then reincubated 60' in NoS plus Nalidixic aicd
- Incubated 60' in S
- Incubated 60' in S plus nalidixic acid

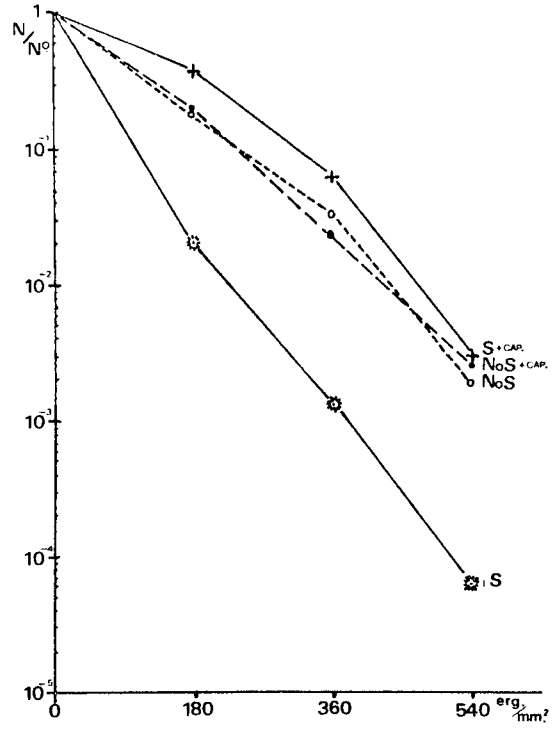

Figure 3.- Effect of cloramphenicol (150 µg/ml) on UV. sensitivity of DA82

- Incubated 60' in S
- Incubated 60' in S plus chloramphenicol
- Incubated in NoS 60'
- Incubated in NoS plus chloramphenicol 60'

D. 7 Liquid Holding Recovery of Salmonella typhimurium

I. Influence of Medium Composition

C.J. Quintans.

After exposure to ultraviolet radiation, some bacterial strains, if incubated for several hours in a non-nutrient liquid medium, display a higher degree of survival than when plated immediately after irradiation. This phenomenon, known as liquid holding recovery (LHR) depends on the activity of the excision repair mechanism, which is the enzymatic process that eliminates fron DNA the pyrimidine dimers produced by UV. light, and fills the remaining gaps with the suitable nucleotides.

The conditions to obtain optimal LHR were studied in the osmotic sensitive mutant Salmonella typhimurium DA82 (1).

In the first place, the effect of the composition of the irradiation medium on LHR was tested. As can be seen in Table I, the irradiation medium is very important for recovery, since changes in the irradiation medium caused large variations in LHR efficiency. When irradiation was carried out in the complex solution M9-20% higher recovery was obtained than when solutions of the same osmolality, but containing one single salt were used. Efficiency of LHR varied also according to the single salt solution employed; KCl was the most effective followed by NH_4Cl, $MgCl_2$ and finally NaCl.

As the medium employed to grow the cells contained glucose, and the presence of this sugar in the recovery medium is inhibitory for LHR (2), experiments were done to investigate whether it was advantageous for LHR to perform washes by centrifugation in order to eliminate completely the growth medium. As can be seen in Table II, washes caused simultaneously a rise in resistance, and a decrease in recovery, and after two washes LHR was abolished.

Finally, experiments were done to decide which was the best composition for the recovery medium. Solutions of different salts were added to samples of irradiated bacteria in M9-20% and incubation was carried out for two hours; viability was then determined for each sample and compared with that of unincubated bacteria. As can be observed in Table III, medium osmolality strongly affects recovery ability (this point is discused in another report). When normal osmolality was employed LHR was dependent upon medium composition; both NaCl and KCl gave a recovery response similar or better than that obtained in undiluted M9 buffer, whereas NH_4Cl and $MgCl_2$ gave a lower level of LHR.

From these results it can be concluded that the conditions for obtaining a good LHR is DA82 are:

1) When bacteria are transferred from growth medium to irradiation medium, washes by centrifugation must be avoided, since that treatment impairs LHR. A good recovery can be obtained by washing the pellet, without resuspension, with irradiation medium and then resuspension of the cells in the same medium.

2) Recovery ability seems to be largely dependent on the composition of irradiation medium; M9-20% proved to be the most adequate among the tested media.

3) The composition of incubation medium is an important factor for recovery. Attention must be payed not only to concentration but to the nature of the salts employed. Optimal recovery ratio is obtained when incubation is carried out in the complex salt medium M9 or in M9-20% plus 0.1 m NaCl or 0.1 m KCl.

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TABLE I

Composition of irradiation medium and LHR

Irradiation medium	Surviving fraction at 0 time (F_0)	Surviving fraction at 2 hours (F_2)	Recovery ratio (F_2/F_0)
M9-20%	6.2×10^{-5}	1×10^{-1}	1612
0.025 m NaCl	2.7×10^{-4}	3.3×10^{-2}	122
0.025 m KCl	2×10^{-4}	7.2×10^{-2}	360
0.025 m NH_4Cl	1.25×10^{-4}	2.7×10^{-2}	216
0.00187 m $MgCl_2$	8.5×10^{-5}	1.5×10^{-2}	176

Aliquot from cells grown to log phase (0.700 OD_{650}) were centrifuged and the pellets were rinsed and resuspended in the solutions listed in table I to OD_{650} about 0.100. All the samples were irradiated with UV. light (540 erg/mm^2). Osmolality was then raised to 250 mOs. by adding 10 x M9 buffer and incubation was carried out at 37ℓ with shaking. After two hours viability was determined by agar plate count.

TABLE II

Preirradiation treatment	Incubation medium	Incubation time (hrs.)	Surviving fraction	Recovery ratio
The cells were harvested by centrifugation and the pellet was rinsed with 20%M9 and resuspended in the same medium	M9 20%	0	1.5×10^{-5}	1
	M9 20%	2	9×10^{-4}	60
	M9 20% plus 0.1 m NaCl	2	4.5×10^{-2}	3000
The cells were harvested by centrifugation; the pellet was resuspended in M9 20%, centrifuged again and resuspended in M9 20%	M9 20%	0	2.6×10^{-5}	1
	M9 20%	2	8×10^{-5}	3
	M9 20% plus 0.1 m NaCl	2	3.2×10^{-3}	123
The cells were harvested by centrifugation; the pellet was resuspended in M9 20%, centrifuged and resuspended in M9 20% and centrifugated and resuspended again in M9 20%	M9 20%	0	4×10^{-5}	1
	M9 20%	2	4.2×10^{-2}	1.05
	M9 20% plus 0.1 m NaCl	2	1.5×10^{-4}	3.75

TABLE III
Efficiency of LHR in different incubation media

Medium composition	Incubation time(hours)	Surviving fraction	Recovery ratio
M9-20%	0	6×10^{-5}	1
M9-20%	2	2.6×10^{-4}	4.4
M9 full strength	2	2.4×10^{-2}	400
M9-20%+0.1 m NaCl	2	3.2×10^{-2}	533
M9-20%+0.1 m KCl	2	3×10^{-2}	500
M9-20%+ 0.1 m NH_4Cl	2	3.5×10^{-3}	58
M9-20%+0.067 m $MgCl_2$	2	1.1×10^{-3}	18

Cells grown in logarithmic phase ($OD._{650}$-0.700) were harvested by centrifugation. The pellet was rinsed with M9-20% and resuspended in the same solution to 0.100 OD_{650}. The suspension was irradiated with 540 erg/mm^2 of UV. light and divided into aliquot parts that received the additions listed in Table III, and were submited to incubation for 2 hours at 37°C with shaking. Viability was determined by agar plate count.

D. 8 Liquid-Holding Recovery of Salmonella typhimurium DA 82

II. Medium Osmolality and Excision Repair

C.J. Quintans.

Excision repair of ultraviolet induced damage occur while irradiated bacteria are incubated in non-nutrient media. The pyrimidine dimers produced by UV. light in DNA are eliminated through the action of a complex enzymatic system that involves, at least, four steps.

1) Incision is catalyzed by specific endonucleases that make a single phosphodiester bond break at or near a pyrimidine dimer.

2) Excision is accomplished by enzymes with UV. exonucleolytic activity, that release the photochemically damaged nucleotides.

3) Reinsertion is due to the activity of DNA polymerases that fill the gaps produced by the preceding step.

4) Sealing is produced by polynucleotide ligase, that catalyzes the formation of a phosphodiester bond between a 5' phosphate and a 3'hydroxyl group.

The capacity to repair UV. damage by incubation in a liquid non-nutritive medium (LHR) was studied in cells of strain DA82 (1) irradiated with UV. (540 erg/mm^2 at 254 nm).

It was observed that the colony forming ability of irradiated bacteria was about 800 times higher when viability was determined after two hours of incubation in a normal osmolality medium (S medium, aprox. 250 mOs.) than when it was assayed immediately after irradiation. When bacteria were incubated in a low osmolality medium (NoS aprox. 50 mOs.), the increase in colony forming ability produced by incubation was only ten times larger (Fig. 1). When NaCl was added to NoS medium, so that osmolality became the same as that in S medium, a rapid increase in recovery rate took place and the same level as the one attained in S medium was reached (Fig. 1). Recovery ability of control Strain DA78 (1) was almost independent from medium osmolality.

As liquid holding recovery is due to the action of the excision repair mechanism (2) several parameters related to the activity ot that enzymatic system were studied.

When the disappearance of pyrimidine dimers from the DNA irradiated bacteria incubated either in S or NoS medium was determined, it was found that there was a remarkable difference between both media in the rate of dimer elimination. Bacteria in S medium excised pyrimidine dimers at a higher rate than cells in NoS (Fig. 2). This difference disappeared, almost immediately, when NaCl (0.1 m) was added to cells incubated in NoS.

As a consequence of the repair process, single strand breaks are produced in the DNA of irradiated bacteria. Those breaks lead to a decrease in chain size that becomes apparent after dissociation of the DNA strands at alkaline pH. The molecular weight in alkaline sucrose of DNA from DA82 cells UV. irradiated and incubated in S and NoS was determined. Cells incubated in either S or NoS showed, after one hour, a similar decrease in DNA molecular weight (Fig. 3). After two hours, completion of the repair process in the cells incubated in S medium produced an increase in DNA molecular weight; on the contrary, the molecular weight of DNA from cells kept in NoS medium remained at the same level observed before (Fig. 3).

From these results it can be concluded that as a consequence of incubation in NoS, DA82 suffers a reversible diminution of its excision repair ability. This impairment appears to affect the excision at the exonucleolytic step and is probably poduced by changes at the cell envelope level caused by the low osmolality of NoS medium.

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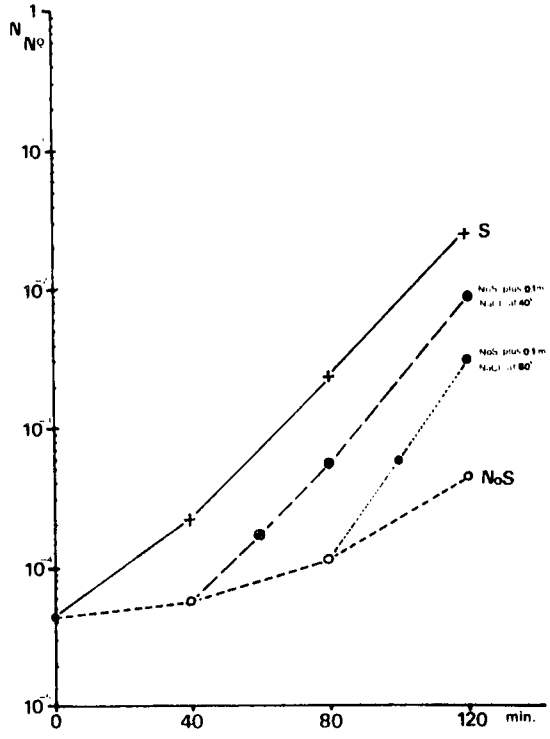

Figure 1

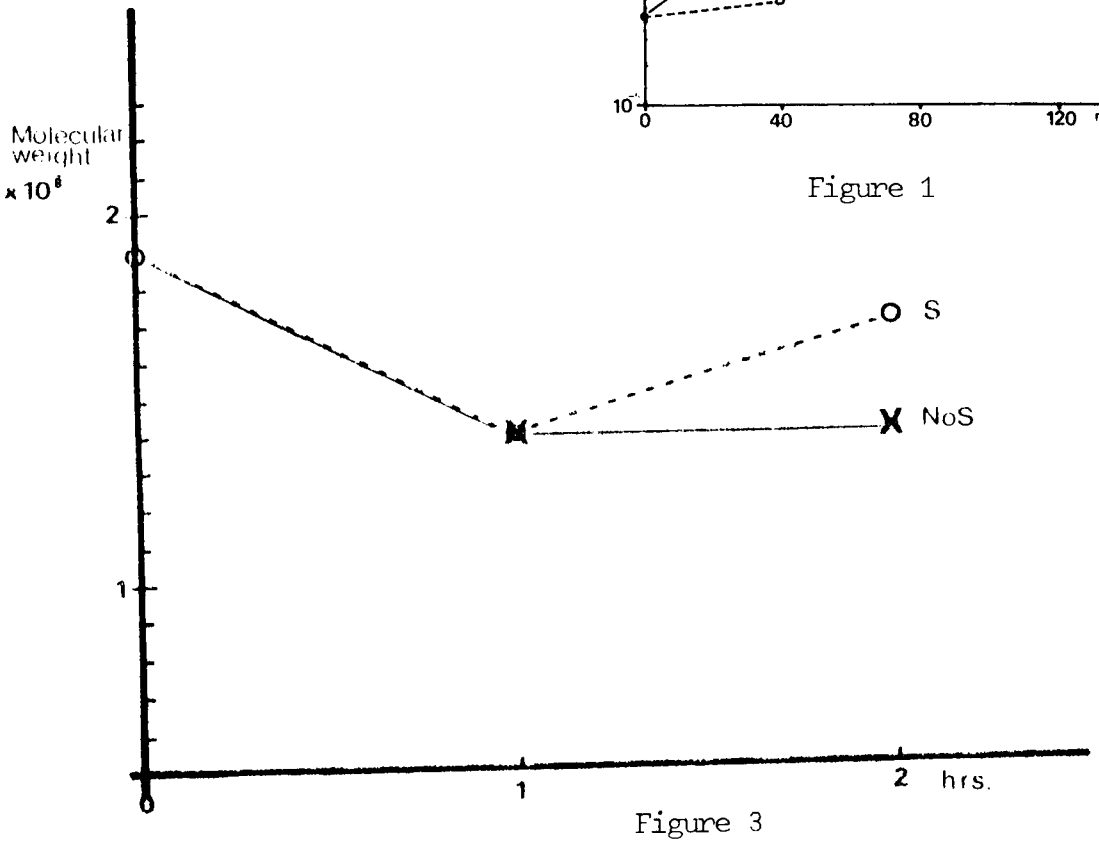

Figure 3

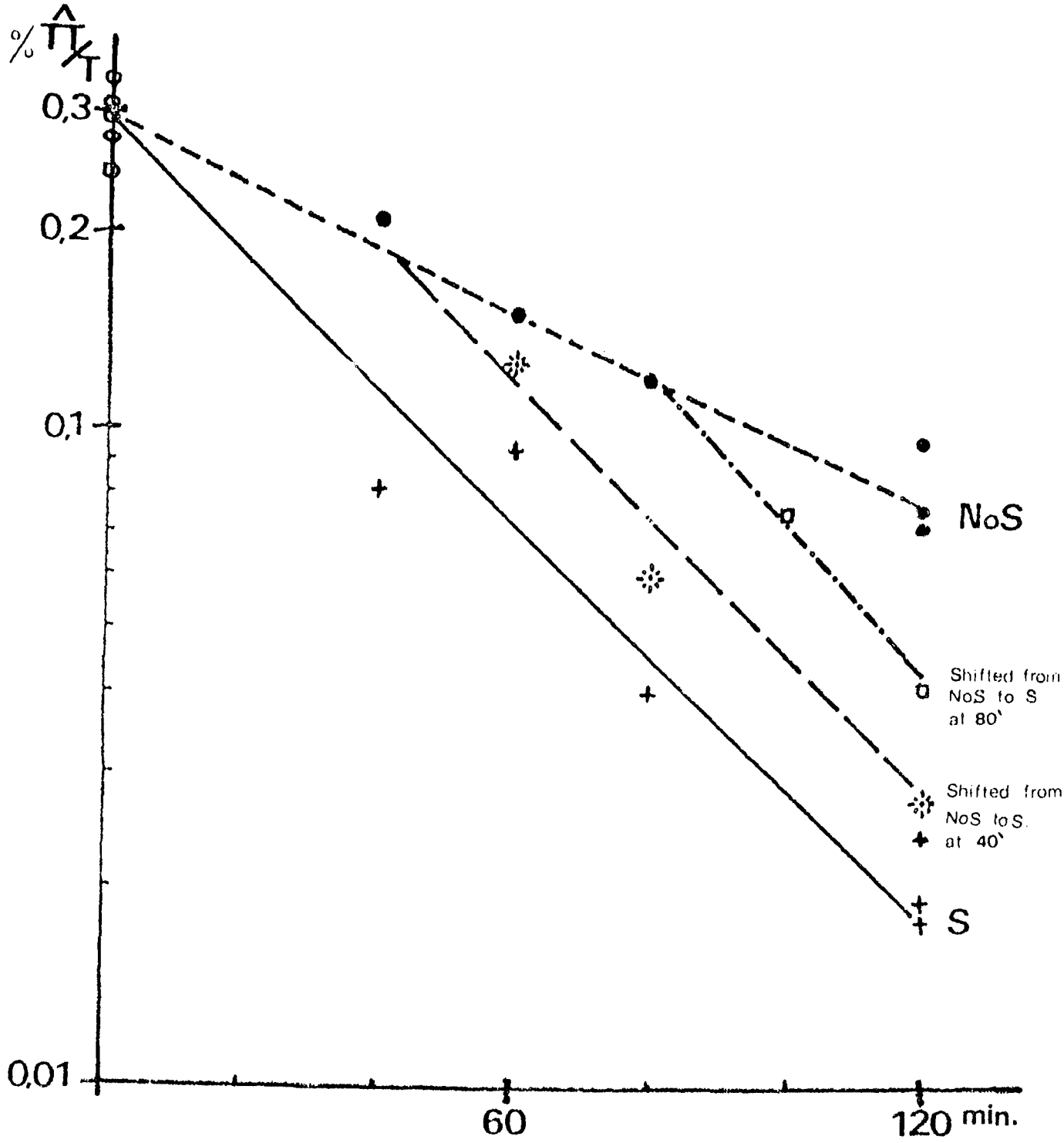
% Π/T
0,3
0,2
0,1
0,01
60
120 min.
NoS
Shifted from NoS to S at 80'
Shifted from NoS to S at 40'
S

Figure 2

D. 9 Liquid Holding Recovery of Salmonella typhimurium

III. Influence of Inhibitors of Macromolecular Synthesis

C.J. Quintans.

When cells of strain DA82 (1) are incubated in NoS (2) medium, there is a decrease in liquid holding recovery (LHR) ability and a simultaneous inhibition in the synthesis of macromolecules (3).

In order to determine whether those two facts are connected, the effect of several inhibitors of macromolecular synthesis on LHR was tested. Nalidixic acid (15 µg/ml) and phenethyl alcohol (0.25% v/v) were used as inhibitors of DNA synthesis; 5-fluorouracil (50 µg/ml) was employed to arrest synthesis of RNA; and tetracycline (50 g/ml), puromycin (67 µg/ml), erythromicin (20 µg/ml) and chloramphenicol (20 µg/ml), were used to inhibit protein synthesis.

LHR experiments were carried out with each one of the inhibitors, with and without the addition of 0.1% glucose. Previous work has demonstrated that in the DA82 system LHR is strongly inhibited by glucose, and that the inhibition is reverted by some agents. Therefore, glucose can be used to uncover differences among the inhibitors employed.

According to the responses observed, the inhibitors can be classified in three groups:

1) Puromycin, 5-fluorouracil and nalidixic acid: they did not affect LHR either in the absence or in the presence or glucose (Fig. 1B).

2) Chloramphenicol: depressed LHR in S medium (2) to the same level as incubation in NoS (Fig. 1C). The same response was observed when glucose was added to cells in S medium (Fig. 1C). Yet even though chloramphenicol and glucose, separately, exhibited similar effects on LHR, they were not able to alter LHR when both were added together (Fig. 1C). This result indicates that the mechanisms of action of chloramphenicol and glucose on LHR are different and antagonistic. Both tetracycline and erythromycin belong to this group, but the responses found at the concentrations employed were weaker than with chloramphenicol.

3) Phenethyl alcohol: incubation with this drug in S medium inhibited LHR to the same level observed in NoS alone; however unlike chloramphenicol, phenethyl alcohol was unable to neutralize the inhibitory effect of glucose.

The results obtained indicate that inhibition of DNA, RNA, or protein sybthesis, separately, does not impair liquid holding recovery. Thus, it would appear that the effect of NoS medium on LHR is not a consecuence of the inhibition of macromolecular synthesis,

The similarity between the effects of phenethyl-alcohol and incubation in NoS is noteworthy. Phenetyl-alcohol has been widely employed as an inhibitor of DNA synthesis in replication studies, but it has been recently demonstrated that its effects are not as specific as previously considered. Acting on the cell membrane as its primary target, phenethyl-alcohol causes the breakdown of the permeability barrier bringing about secondary effects such us inhibition of synthesis of macromolecules, depression of respiratory activity, facilitated entrance of molecules which are normally excluded by the cell membrane, and inhibition of repair of radiation damage. Incubation of DA82 in NoS medium gives rise to similar effects; therefore, phenethyl-alcohol could perhaps help to undestand the molecular basis of the behavior of this interesting mutant.

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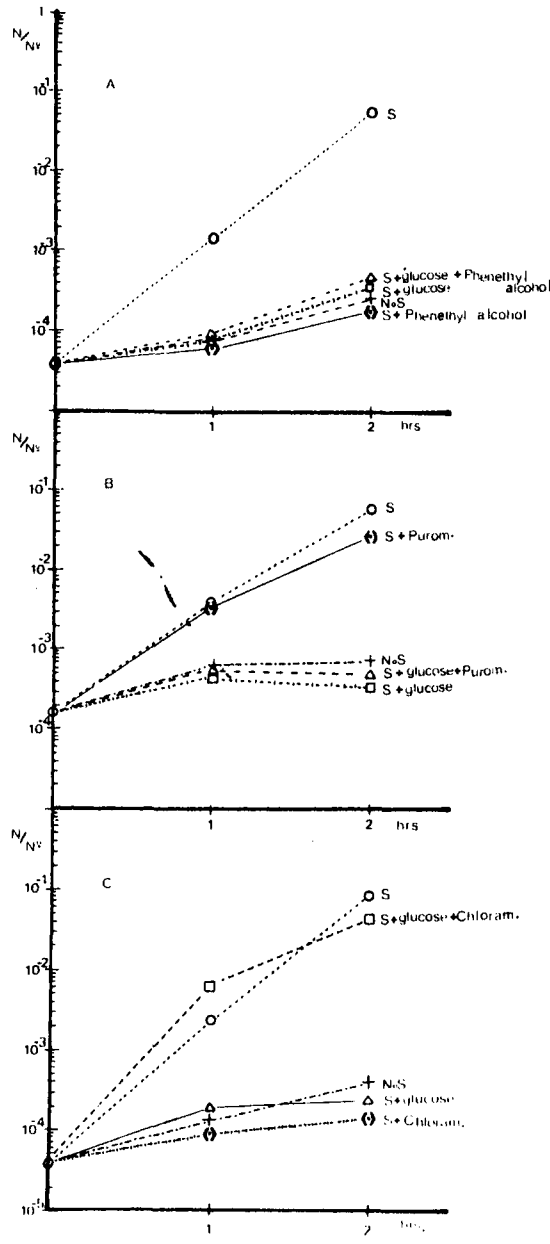

D. 10 Thermosensitive Mutant of *Salmonella typhimurium* with Alterations in Cell Division

A.T. De Micheli and D.N. Antón

At least four alterations have been identified in strain DA138: sensitivity to deoxycholate (DOC-3 mg/ml), abnormal cell shape, sutolysis in buffer Tris-ClH pH 8.5 (37°C), and thermosensitivity.

In crosses between an auxotrophic derivative of DA138 and donor strains belonging to different Hfr types, the mutation conferring thermosensitivity was localized between markers metA(128 min.) (1) and leu (3 min.). Employing two Hfr strains that transfer the chromosome from about the same point of origin but in opposite directions, the location of the thermosensitive mutation (tms) was confined to the segment 0-3 min.. Finally, 254 leu^+ transductants selected in a cross between a thermosensitive recipient and phage grown on a thermoresistant ara^- donor strain were analyzed for unselected markers. On the basis of the frequencies of cotransduction observed: leu-ara (53.1%), leu-tms (11.8%), and leu-ara-tms (1.2%) the following order was established for those three markers: ara-leu-tms.

The location of the tms mutation suggests that it belong to divC, a gene already identified in S. typhimurium (2). This assumption is corroborated by the finding that, like the only divC mutant as yet described (2), the tms mutation of DA138 is able to restore the smooth phenotype to histidine regulatory mutants.

Several recombinants from the crosses and transduction reported were analyzed for the other alterations present in DA138. Segregation was observed for autolysis, sensitivity to DOC, and thermosensitivity. Thus, at least three differente genes are mutated in DA138. No information on the genetic control of cell shape is yet available.

Preliminary results indicate that the gene involved in sensitivity to DOC is located near metA (128 min.). Furthermore, it has been shown that the mutation involved in autolysis does not belong to the same gene as the envD mutations that produce a similar phenotype in other envelope mutants of S. typhimurium (3).

Since the study of the alterations produced by the tms mutation could be influenced by the presence of other envelope defects in DA138, the tms allele was transferred to a wild type strain. Phage grown on DA138 was used to transduce an appropiate leu^- recipient and strain DA470 was obtained. It shows the following properties: DOC-resistance, normal autolysis and normal bacillar shape. Yet, it cannot grow on nutrient agar at 43°C, so strain DA470 has received from DA138 only the altered response to temperature. Strain DA470 was employed to study the effect of temperature on growth and cell division.

When cells of DA470 growing in nutrient broth at 32°C are transferred to 43°C, there is a very rapid arrest of cell division. However, growth is not inhibited since the optical density of the culture increases at the same rate as the control at 32°C (Fig. 1). It can be seen by phase microscopy that long filaments are formed. These filaments do not appear to be segmented.

The inhibitory action of temperature on cell division is reversible. When filaments formed at 43°C are transferred to 32°C they start dividing at a very fast rate almost immediately; once the titre of the control at the moment of the shift is reached, cell division continues at a normal rate (Fig. 2).

Recovery by transfer to 32°C occurs even in the presence of chloramphenicol (32 µg/ml), although in this case fewer divisions take place (Fig. 2).

Septation can be induced in some E. coli and S. typhimurium mutants by an increase in the osmotic pressure of the medium (4), (2). Addition of NaCl (0.25 M, final concentration) to filaments of DA470 kept at 43°C, stimulates cell division. The effect of NaCl is partial since the number of viable cells reached is much lower than that obtained by transfer to 32°C. Furthermore, the increase in cell number is not as fast as in that case (Fig. 2).

The results obtained suggest that some product necessary for cell division accumulates during growth at 43°C and allows the fast division cycles observed after transfer to 32°C. Some of these divisions do not require protein synthesis to proceed. It is concluded that the alteration carried by DA470 affects one of the final steps in the process of cell division.

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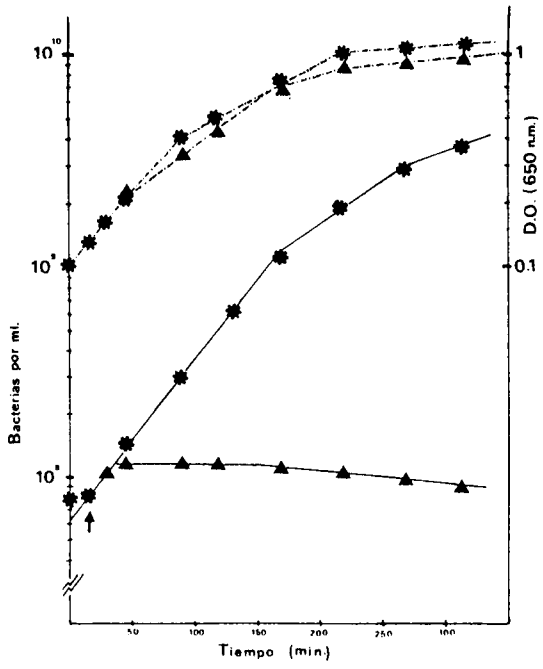

Figure 1.- Behavior of DA470 in nutrient broth at 32°C (✱) and at 43°C (▲). At 30 minutes (↑) an aliquot of the culture growing at 32°C was transferred to 43°C
-------- O.D. (650 nm). —— Viability

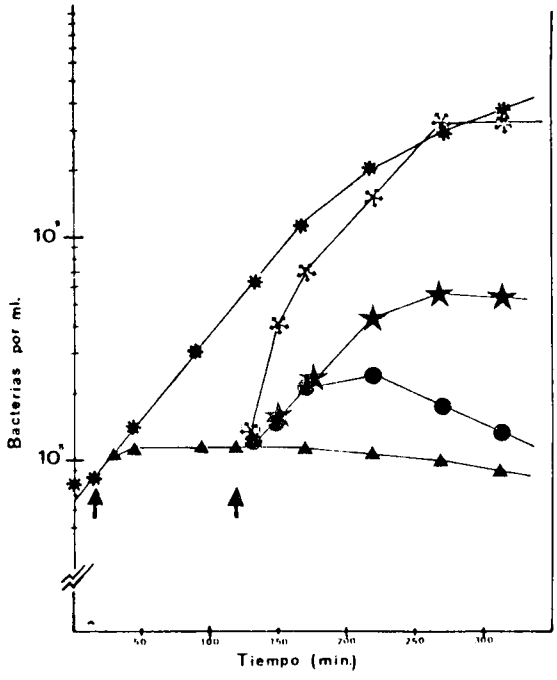

Figure 2.- Cell division of DA470 in nutrient broth at 32°C (✱). At 30 minutes (first arrow), an aliquot of the culture growing at 32°C was transferred to 43°C(▲).After 120 minutes (second arrow) samples of the culture at 43°C were transferred to(✲)32°C, (●) 32°C + 32 µg of chloramphenicol and 43°C + 0.25 M NaCl (★).

E. SOMATICS EFFECTS OF IONIZING RADIATION (ONCOLOGY)

E. 1 · Evolution of Viral Infection in Whole Body Irradiated Animals: I. Behaviour of Passanger Virus

J. Mayo and J.H. Lombardo.

Endogeneous infection is one of the most important early consequence of whole body irradiation in human beings and experimental animals (1). Based on this statement a line of research was developed with the purpose of stablishing the importance of viruses as a cause of infection in irradiated animals.

Previous work has demonstrated that immune animals died as result of virus infection if animals were inoculated with the agent after being irradiated (2).

The foot and mouth disease virus which was used in this experiments was adapted to the host animals after severals transfers into whole body irradiated adult hamsters. Therefore the adapted virus was converted in to a new factor of disease in a naturally immune animal (3).

As a result of these experiments the study was extended to viruses which possessed the property of transforming normal into neoplastic cells. Viruses with the property of inducing malignancy have been shown to produce tumors in whole body irradiated animals (1).

Another study demonstrated that a passanger virus occurring in a hamster tissue-culture cell-line, BHK 21 cl 13 shows its infectious and oncogenic properties after the host cells have been passed through whole body irradiated animals (4-5-6-7).

The cells having the oncogenic BHK virus produced in hamsters a undifferenciated sarcoma. The sarcoma cells were subsequently cultured and a cell line, BHK 169, was obtained. A cell-free suspension was made from animal and culture cells, the virus recovered and its oncogenic properties investigated (7).

The cell-free suspension was centrifuged and the pellet and supernatant separated into several fractions by column chromatography. The fractions were analized chemically and by radioisotope labelling. The oncogenicity was determined by testing the property of transforming animal and culture cells.

The tissue-culture cell-line BHK 169 was used to verify the production of reactions by neoplastic cells. Tissue culture cells in the order of 2.10^7 were placed in diffusions chambers with millipore filter walls of 220, 50 and 10 nm pore-diameter. The chambers were implanted in the peritoneal cavity of adults hamsters and tumor induction investigated.

The same procedure was followed "in vitro" by placing diffusion chambers in contact with primary cultures of hamster peritoneal and embryo cells.

With the above mentioned procedures two fractions were isolated. A fraction of molecular weight 15.10^7 daltons corresponding to the viral agent and another of a molecular weight 3.10^6 daltons corresponding to the virus RNA.

When the diffusion chambers were filled with cultured malignant cells both fractions caused cellular transformation in animals and in peritoneal and embryo primary cell-cultures as well.

It was also demonstrated that the original passanger viruses induced transformation of BHK 21 cl 13 cells cultures after been transferred several times in whole body irradiated animals. These results show a change in the expression of the original viral genome.

In brief the presented data shows the oncogenic property of a corona-virus existing as passanger virus in the BHK 21 cl 13 cell line. This property is clearly demonstrated after the virus-infected cell was transferred through whole body irradiated animal.

The isolated virus genome was able to transform cultured and non-cultured cell into malignant cells, this would be a natural mechanism which would not need the presence of the entire virus.

We should like to point out that the carrier of this virus, the BHK 21 cl 13 cell, is used to prepare vaccines against foot and mouth disease in cattle, and widely used in research in the field of cell and virus biology.

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E. 2 Evolution of Viral Infection on Whole Body Irradiated Animals.
II. Behaviour of Viruses with Oncogenic Activity.

J. Mayo

In a further analysis of this research line we investigated the possibility of a change in the behaviour of a oncogenic virus after passing the host cell through a whole body irradiated animal (1-2-3).

Spontaneous mammary tumor cells obtained for C_3H/Ep mice were inocculated intraperitoneally into animals of the same strain. The animals were previously whole body irradiated with a dosis of 400 rads.

At the 5^{th} transfer in irradiated animals a change in the spread of the tumor in the peritoneal cavity was noted. The isolated nodules observed previously were replaced by many small tumors and hemorrhagic ascitic fluid with suspended neoplastic cells.

At present, after eleven transfers in whole body irradiated animals the tumor shows the same characteristics. The cells from the peritoneal nodules were easily cultured as primary culture and a vitro line will be probably obtained. The aim of this study is to investigate the behaviour of virus and host cells after inoculation into whole body irradiated animals.

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F. PATHOLOGY

F. 1 . Radiation Effects on Rat Epidermis: Sulphydryl and Disulphide Groups

A.C.C. Frasch, M.E. Itoiz, H.E. Volco and R.L. Cabrini.

Questions are still to be answered concerning radiation effects on SH and SS groups. Csermely, Panfilis and Santani (1) report that SH withstood 100 to 1000 rads with no variation, whereas Guerrier, Carayée and Varenne (2) report a decrease, after a 7000 rads dose of cobalt-therapy in human epidermis.

In our laboratory, we developed a method for quantitative evaluation of the cellular distribution of SH and SS groups by the microspectrophotometric scanning of sections stained with the Mercury-Orange technique (3), and it seemed useful to apply it to the study of normal and irradiated epithelium.

The dorsal skin of seven newly-born Wistar rats was irradiated through 300 and 1200 μ wide lead slits. The source was an X-ray PW 1120 Philips diffractor, at 30 kV and 20 mA with a copper-anode tube and a nickel filter from which a monochromatic beam of 1.54 Å and 8 keV energy was obtained. A single dose of 16 krads was given for 5 min.

Serial sections were cut and three adjacent ones were stained according to the following techniques: (1) haematoxylin-eosine (H and E); (2) Mercury-Orange, for SH groups demonstration; (3) Mercury-Orange, previously reduced in 0.4 M thioglycolic acid, for SH and SS demonstration. The thickness of basal, spinous, granular and horny layers was evaluated in normal and irradiated areas of the H and E sections.

The sections were quantitated by microspectrophotometric scanning in a Cytoscan Zeiss MSP with programmable scanning plate. The general method used followed the recommendations of earlier reports (4).

The greatest concentration of the SH groups in the basal layer decreases towards the superficial strata. The quantitation of reduced preparations yields higher values because they show SH and SS groups jointly. It is in the horny layer that they achieve the maximum total quantity.

The total SH content in the irradiated epithelium increased with respect to the normal value; the increment was higher in the spinous layer (Figure 1 (A)). Conversely, the mean concentration markedly decreased in the basal and spinous layers, (Figure 1 (B)), where the greatest SH concentration for normal epithelium is to be found. The bigger the irradiated area, the clearer the differences.

The total content of SH + SS increased in the irradiated epithelium. The ratios between experimental and control values are similar to those obtained for the SH groups alone (Figure 2 (A)). The SH + SS mean concentration diminished in the basal and spinous layers with minor variations in the upper strata, there being, however, a remarkable decrease in value of total mean concentration of the whole spithelial thickness (Figure 2 (B)).

Striking variations appear in the SH and SS groups, in the granular and horny layers, 4 days after irradiation. Newly-born-rat basal epithelial cells take approximately 3 days to reach the granular layer. This implies either that the new germinal cells have an altered metabolism, or that the underlying dermis injury is such that the epithelial cells are prevented from incorporating the substances they require for their metabolism.

The metabolic alterations, coupled with the decrease in the mean concentration of SH and SS groups and the ultrastructural alterations observed in horny cells, would allow the presumption that, although accelerating the keratinizing process, the irradiated epithelium modifies the horny substance both in quality and in quantity.

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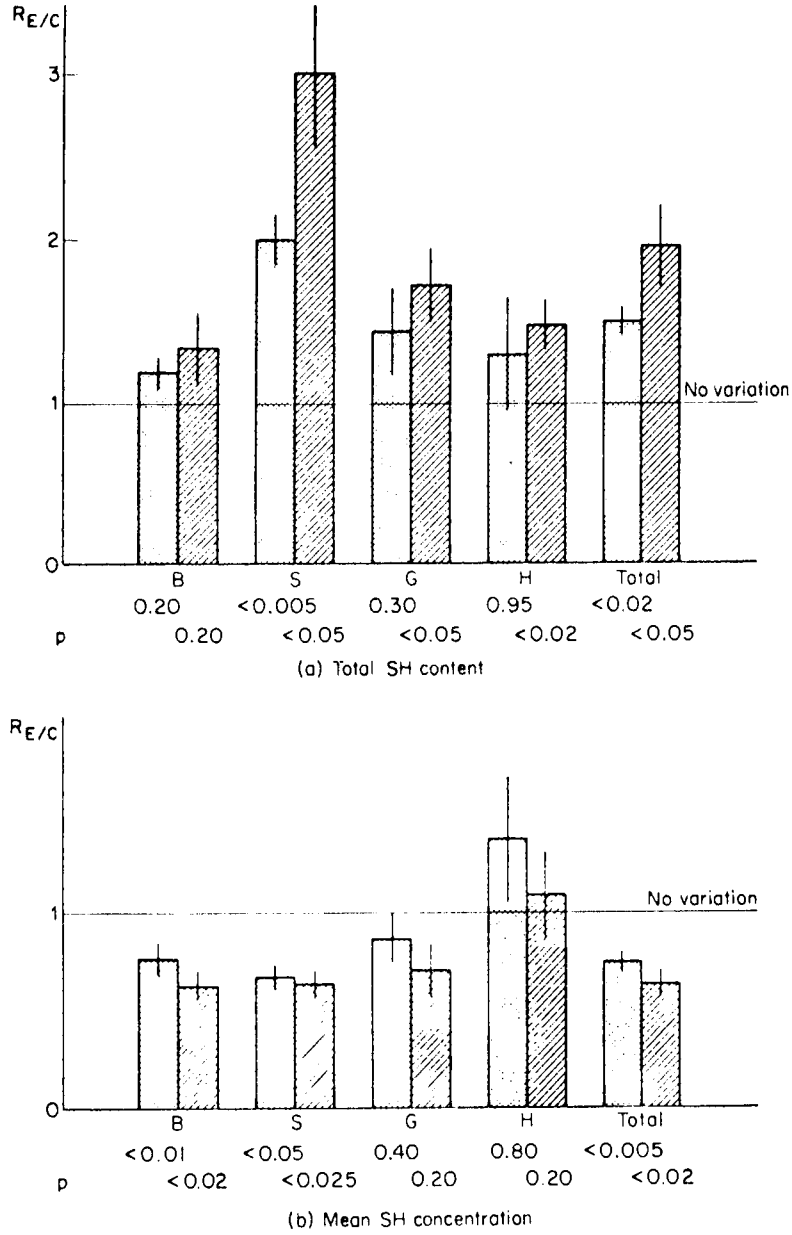

Figure 1.- Total (a) and mean (b) values of SH (Mercury-Orange method without reduction). Ordinates show the ratio between experimental and control values. Results corresponding to 2 cm x 300 μ (....) and 2 cm x 1200 μ (////) irradiated area are shown for each epithelial B: basal layer; S:spinous layer; G:granular layer; H: horny layer; p: probability ("t" Student test with paired data).

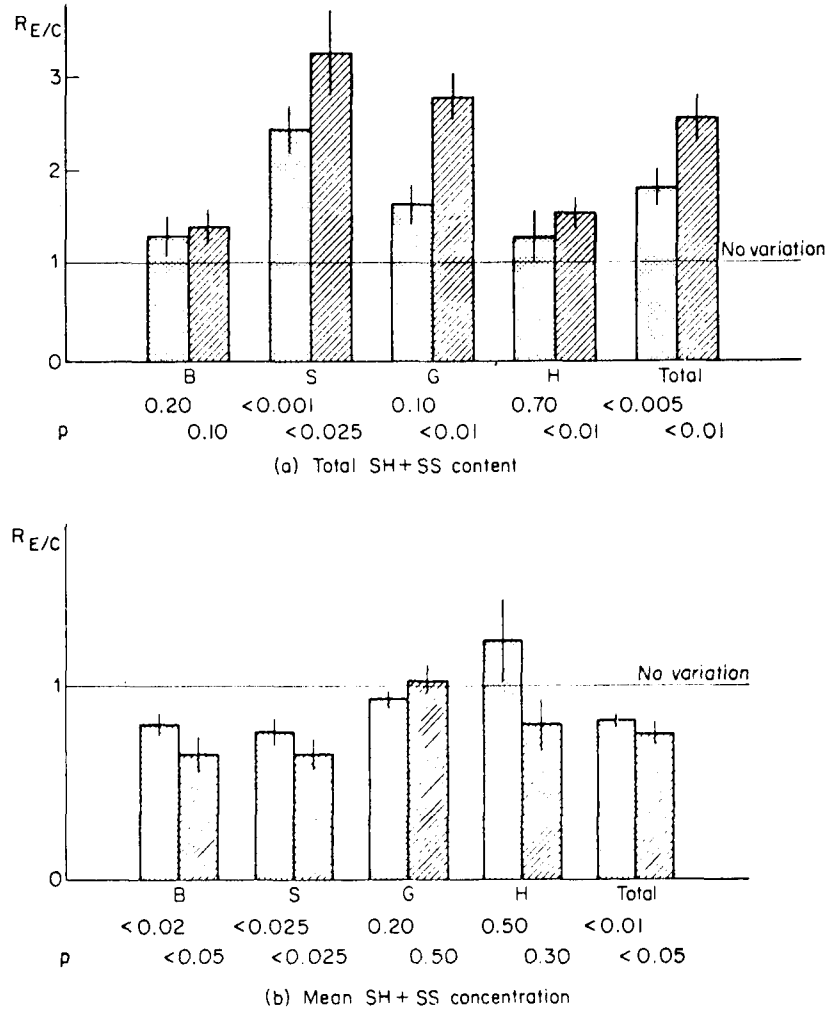

Figure 2.- Total (a) and mean (b) values of SH + SS (Mercury-Orange method with reduction). Ordinates show the ratio between experimental and control values. Results corresponding to 2 cm x 300 μ (...) and 2 cm x 1200 μ (////) irradiated area are shown for each epithelial strata. B: basal layer; S: spinous layer; G: granular layer; H: horny layer; p: probability ("t" Student test with paired data).

F. 2 Radiation Effects on Rat Epidermis : Succinic Dehydrogenase, Lactic Dehydrogenase and Glucose-6-Phosphate Dehydrogenase Variations as Function of Age and Lesion Localization

M.E. Itoiz and A.C.C. Frasch.

In a series of previous reports reference is made to different aspects of the epithelial response to high doses of X-ray delivered to the tail epidermis of new-born rats.

In addition to these epidermal changes, there have been repeated reports on glycolytic metabolism modifications showing a constant decrease of enzyme activities related to the Krebs cycle and an increase of those associated with the pentose shunt (1-3).

Based on this viewpoint, it seemed interesting to establish the influence of other factors such as age or structure of the irradiated areas, which might modify the degree of enzyme response.

Ten Wistar rats, 3 and 12 days old, were irradiated on the right sole (a hairless area) and on the distal third portion of the tail. The left sole and the proximal two thirds of the tail were used as non-irradiated controls.

The animales were sacrificed 4 days after irradiation.

The sections were stained with the usual hematoxylin-eosin (H-E) technique and with histochemical techniques for demonstration of glucose-6-phosphate dehydrogenase (Gl.6.ph Dh), succinic dehydrogenase (SDh) and lactic dehydrogenase (LDh) (4-5).

The epidermal enzyme activities were determined with a microspectrophotometer provided with automatic stage (Zeiss Cytoscan).

The total enzyme content (TEC) of SDh and LDh increased in all cases studied, except in the sole of 7 days old animals. The mean concentrations (MEC) decresed, indicating a smaller availability of both enzymes per unit volume. In all cases the most important variations occurred in the tail and in the younger animals; a phenomenon coincident with the morphologic alterations (acanthosis).

Gl.6.ph Dh showed a reactive increase of both the TEC ans the MEC in all studied conditions. As happens with the other tested enzymes, the largest variations were found in the younger animals. Although the total content of this enzyme is higher in the tail than in the sole, the weaker acanthotic reaction in the latter is responsible for the higher values per unit volume (MEC) in that region.

The present data confirmed previous results regarding the decrease of SDh and LDh and the increase of Gl.6.ph Dh (2-3).

Young animals, suffer larger morphologic and enzyme alterations of the epidermis when exposed to radiation.

The differences observed between the sole and the tail seem to indicate that the presence of hair follicles in the tail increases the degree of damage produced.

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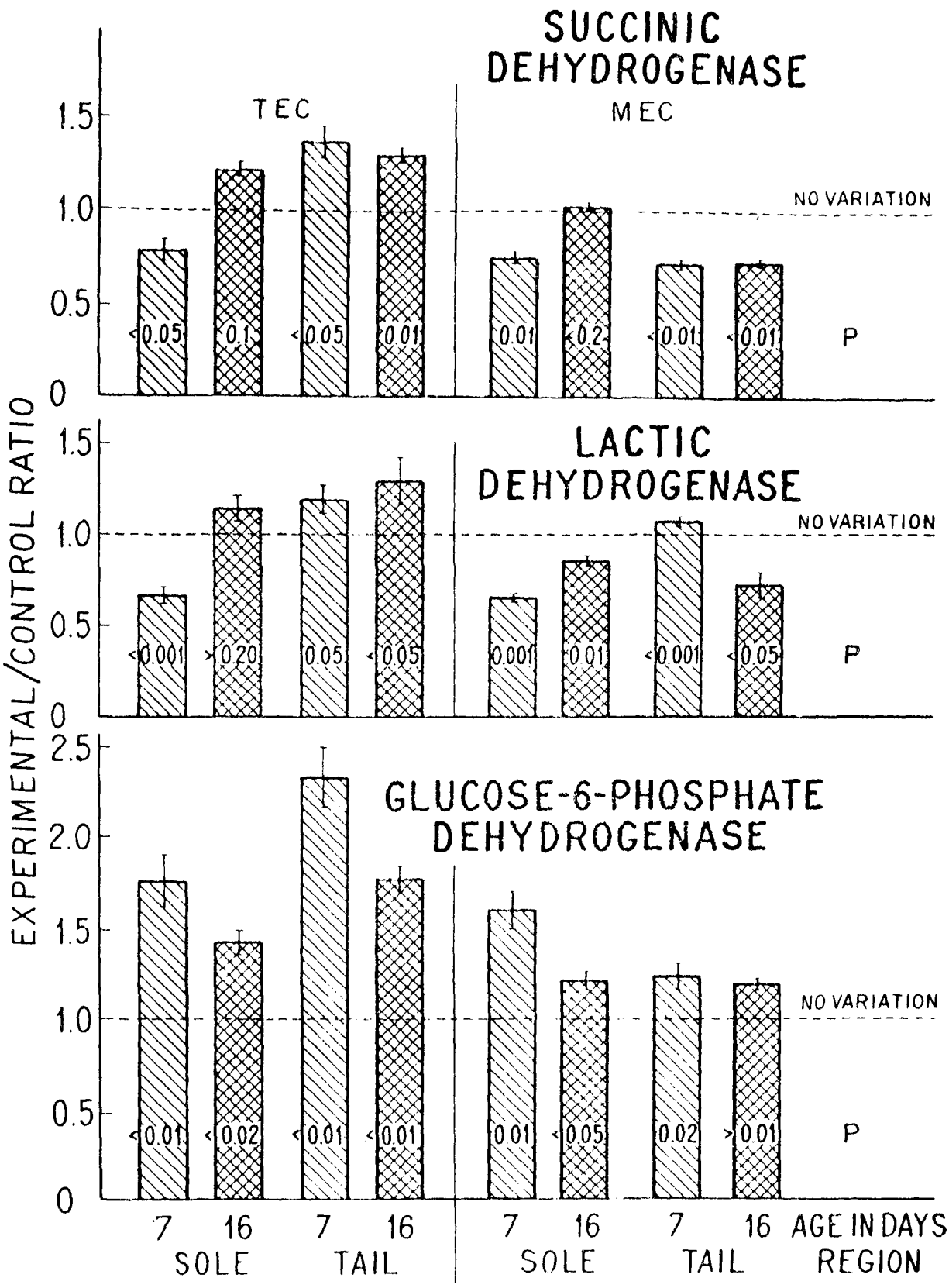

Figure 1.- Total enzyme content (TEC) and mean enzyme concentration (MEC) of succinic dehydrogenase, lactic dehydrogenase and glucose-6-phosphate dehydrogenase. Ordinates show the ratio between irradiates and control values. p: probability (Student's test using paired data).

F. 3 Histoenzymic Changes in the Epidermis of Irradiated Wounds.

I.B. Gimênez, M.E. Itoiz and R.L. Cabrini.

The healing process of experimental wounds constitutes a suitable model for the study of an actively proliferating epithelium under different types of injury.

Certain metabolic aspects of the proliferating epithelium at the margin of normal rat skin wounds were previously analysed in our laboratory. Krebs cycle enzyme activities decreased in the acanthotic epithelium and their microspectrophotometric values were in inverse relation to increasing epithelial thickness. This decreased activity of the Krebs cycle coincided with an increase in the pentose shunt metabolism.

In the present study the epithelial enzyme response in irradiated wounds was analyzed using the previously mentioned animal model.

1 cm^2 square wounds in the aminal's side were made in the dorsal and lumbar skin of adult Wistar rats. The lumbar wound was locally irradiated with 6000 rads of X rays. The dorsal wound which was under the total body shield was used as a control of the normal healing process.

Irradiation was performed a few minutes after wounding. Groups of 5 animals were sacrificed after 7 and 14 days. Unfixed specimens obtained from dorsal and lumbar wounds of each animal were mounted in a single block and sectioned in a cryostat. Histochemical demonstration of Succinic Dehydrogenase (SDh) and Glucose-6-phosphate Dehydrogenase (Gl-6-ph Dh) were performed according to Nachlas et a. (3) and Hess et al. (4).

Enzymatic activity was quantitated by means of a Zeiss Cytoscan microspectrophotometer, with a scanning stage. Using a 1 μ diameter spot, 6 scans from basal to superficial layer were performed in the acanthotic epithelial margin of each wound. The following data were obtained: total enzyme content of the epithelium (TEC) epithelial thickness (or scanning length : ET) and mean enzyme concentration (MEC) (by dividing TEC by ET) which expresses enzymatic activity per unit volume of tissue. The ratio between values obtained from irradiated and control wounds of each animal was used to compare the irradiation effects in different specimens.

On the 7th day of healing process, the irradiated wound showed a decreased activity of both enzymes explored and a weak proliferative response (Figure 1-2). On the 14th day Gl.6.ph Dh of the irradiated wounds exhibited an increased value of TEC but MEC, although greater than the value of the 7th day did not reach normal levels (Fig. 1-2).

Since enzymatic patterns at the 14th day were similar to those observed at the 7th day in control wounds, it would appear that irradiation produces a delay in the metabolic variations which normally occur in healing epithelium. This delay would be partially caused by alterations in the epithelial nutrition because a lower initial amount of vessels was found in the irradiated wounds, under the same experimental conditions (5). This diminished vascularization was found to be compensated during the healing period.

Further experiments are being conducted to study the epithelial behaviour during longer healing periods, in order to investigate whether the applied dose only modifies the schedule of events or if the epithelium suffers later alterations which may prevent complete regeneration.

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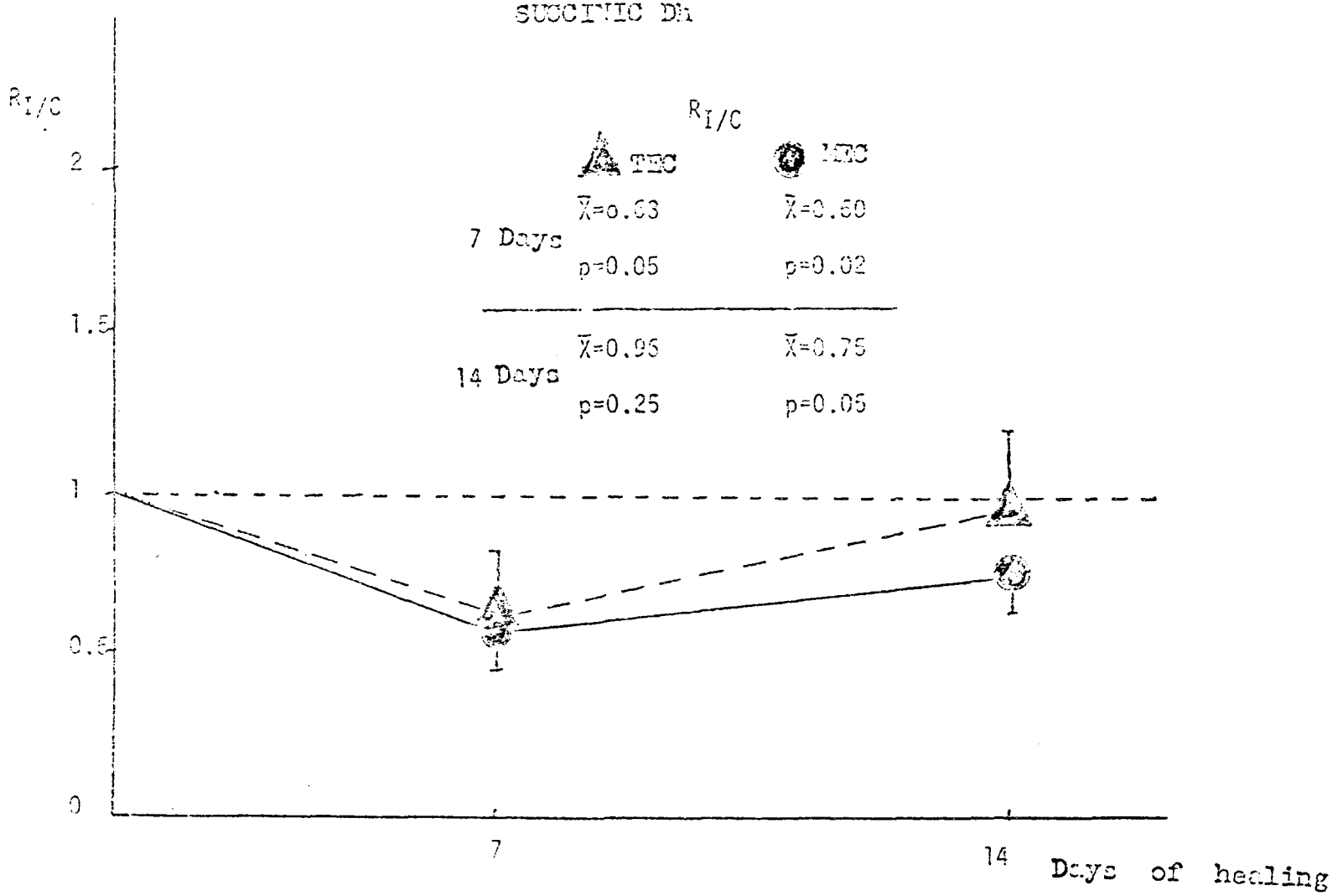
SUCCINIC DH
$R_{I/C}$
2
1.5
1
0.5
0
7
14
Days of healing
$R_{I/C}$
TEC
NEC
7 Days
$\bar{X}$=0.63
p=0.05
$\bar{X}$=0.50
p=0.02
14 Days
$\bar{X}$=0.95
p=0.25
$\bar{X}$=0.75
p=0.05

Figure 1

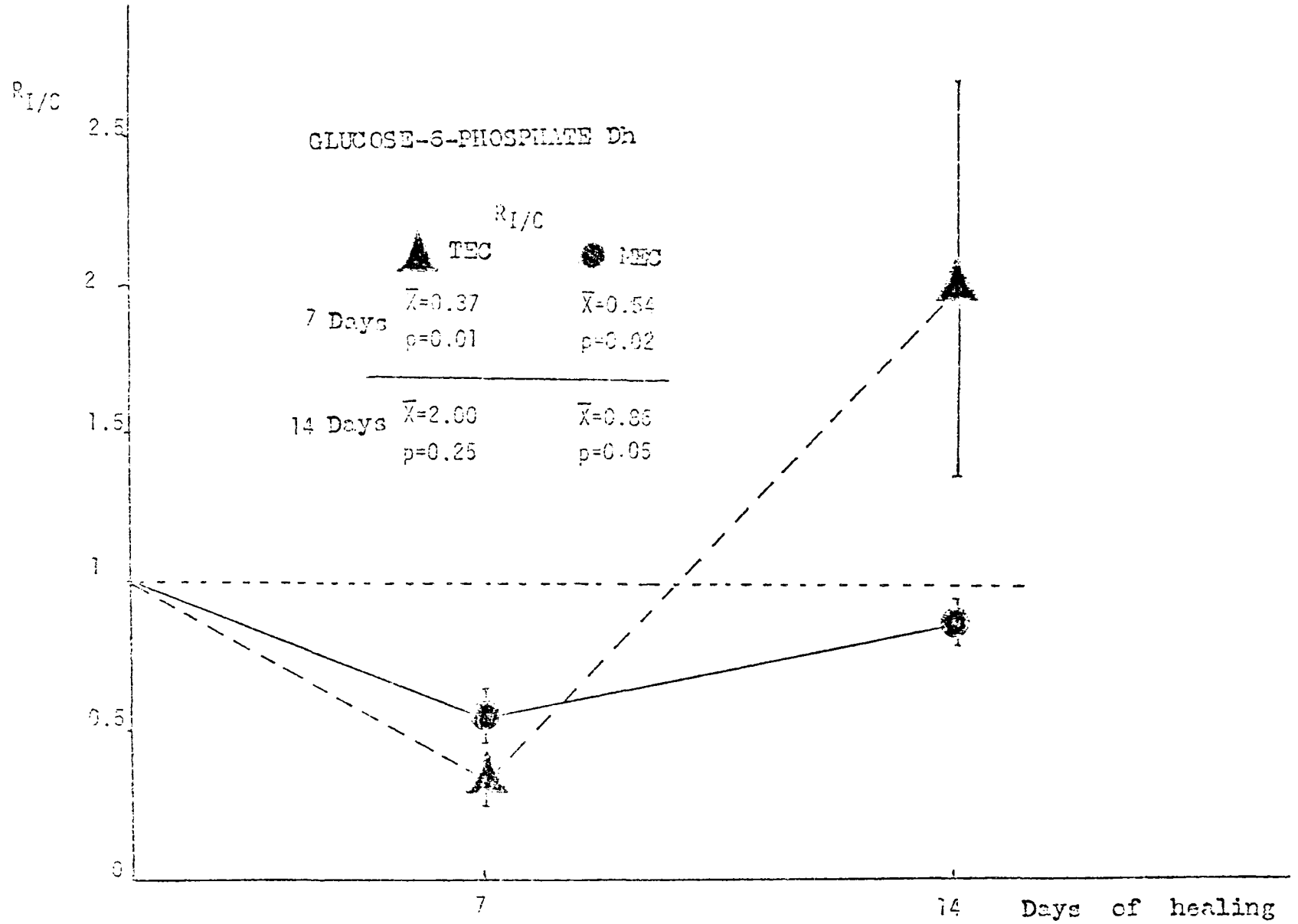
$R_{I/C}$
2.5
2
1.5
1
0.5
0
GLUCOSE-6-PHOSPHATE Dh
$R_{I/C}$
TEC
MEC
7 Days
$\bar{X}$=0.37
p=0.01
$\bar{X}$=0.54
p=0.02
14 Days
$\bar{X}$=2.00
p=0.25
$\bar{X}$=0.86
p=0.05
7
14
Days of healing

Figure 2

F. 4 Ultrastructural Localization of Acid Phosphatase in Normal and X-Irradiated Epidermis

B.M. de Rey, M.E. Itoiz, A.C.C. Frasch and R.L. Cabrini.

At the ultrastructural level, Ac Pase, like other hydrolytic enzymes, has been classically localized in the epidermis and described as a lysosomal enzyme (1-2). However, its localization remains under discussion.

Itoiz et al. (3) reported that the histoenzymatic quantitation at optical level of Ac Pase in irradiated epidermis showed a remarkable increase in activity in the granular layer. It seemed interesting to take advantage of the model already tested at optical level in order to apply it to ultrastructural aspects of Ac Pase both in normal and irradiated epithelium.

At the ultrastructural level, the greatest concentration of enzynatic activity was found at the granular layer, both in normal and irradiated epithelia, a fact which confirms optical level findings.

The lower spinous layer showed no reaction but in the upper zone a fine diffuse intracytoplasmic precipitate was observed.

In the granular layer, the free reaction product in the cytoplasm was found in greater quantities than in the spinous layers. In the transition zone towards the keratin there was an almost continuous front of intercellular reaction which in some areas showed a keratinosome-like striation.

There was an outstanding reaction in the extracellular spaces of the keratin layer. Intracellular reactive zones were also observed and interpreted as invaginations of keratinyzing cells (4).

Enzymatic distribution was alike in mornal and irradiated epithelium, there being a remarkable reaction increment in the latter.

The precipitates in granular cell cytoplasm were much more abundant than in the controls.

The horny layer also showed an increased activity in the extracellular spaces.

Lysosomes were not found in the basal layer of the irradiated epithelium. Conversely, electron dense, radiation induced, perinuclear vacuoles, as previously described (4) were clearly observed. In the histochemical preparations under study a lead precipitate was observed either in these vacuoles or laid on their surface.

Lysosomes have not been described in the epithelium in significant quantities (2), even through it is possible to find them in the basal and spinous layers.

The numerous keratinosomes found in the subcorneal layers have been equated to a form of epithelial lysosomes, in relation to the keratinizing process, we found Ac Pase positive striated structures in the intercellular space of the granular layer. Presumably, the keratinosomes pour their contents into the extracellular space.

Epithelial Ac Pase activity has been related not only with an eventual role in the keratinization mechanism, but also with phagocytic activity.

At the beginning, the phagocytic induction does not increase the keratinocytic activity; this occurs a few hours later, parallel to the reaction increments of the Ac Pase surrounded by membranes (2). However, the heterolysósomes would not account for the greater enzymatic activity found in the upper epithelial layers.

In our experimental model, irradiation originates and increased keratinization linked to a rising enzymatic activity. Similar increments have been described for this enzyme in cells which increase their catabolism or which suffer a regression. It can be inferred from the data collected that under these conditions the epithelial response to irradiation is a hyperkerathotic process coincident with a speeded up intracellular destruction.

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F. 5 Quantitative Modifications of Proteins and Nucleic Acid Content in Reactional Acanthosis of Irradiated Epidermis

C.J. Conti, I.B. Gimênez and R.L. Cabrini.

The purposes of this study are to determine the quantitative modifications of proteins and nucleic acids in irradiated epidermis, and to analyze the feasibility of taking these results as reference patterns in the quantitative study of the enzymes determining their specific activity, in order to differentiate the actual modifications from those produced by dilution during the processes of hydropic degeneration.

Two centimeter areas of the mid-third of the tail of 4-to-5-day-old Wistar rats were exposed to local X-irradiation.

For measurements of Proteins and total Nucleic Acid, sections were stained with Naphtol Yellow S and Gallocyanine Crom-Alum respectively for measurements of DNA content the Feulgen procedure was employed using a predetermined hydrolisis time of 35 minutes at 28°C in HCl5N.

The quantitative determinations were carried out in a Zeiss, Cytoscan microspectrophotometer. Microspectrophotometric scannings from basal layer to the surface were performed in each section.

In order to avoid mistakes due to variations either in the thickness of sections or in the histochemical reactions, the irradiated area and the normal area of each case were measured in the same section and a ratio between the two values was used.

The amount of proteins and of total nucleic acids in 8 Krads irradiated epidermis increased concomitantly with reactional acanthosis. However, the concentration of these substances decreased as from day 3 post-irradiation (Fig. 1 and 2).

A similar behaviour was observed when using 4 Krads.

In the 8 Krads irradiated epidermis the DNA concentration decreased as from day 2 post-irradiation, reaching a 70% decrease on day 4 and remaining unchanged until day 6 (Fig. 3). The decrease of DNA concentration was weaker for 4 Kr. (Fig. 4). As far as the total quantity of DNA is concerned, a different behaviour was reported, since on day 2 it decreased for 8 Kr. while it increased for 4 Kr.

A statistically significant negative correlation was found between concentration and post-irradiation time and between concentration and thickness for DNA, for proteins and for total nucleic acids. A significant positive correlation between thickness and post-irradiation time and between total quantity and thickness for proteins and total nucleic acids, was also founda (Table 1).

Histological histometric and ultraestructural (1-3) studies of irradiated skin have shown that the reactional acanthosis is due to an increase in the cellular volume, possibly originated through aqueous imbibition. This would in turn be due to an increased vascular permeability, since plasmatic proteins were detected in the epidermis (4).

However, biochemical studies on 28000 rep-irradiated skin have not reported a considerably increased water content (5). In some cases a decrease was observed for a dose of 3000 rep. For the same dose a decrease has also been demonstrated

up to 45% of the DNA and RNA concentrations, whereas the total N protein has not shown any significant changes (6). Our data illustrates this problem from the histochemical point of view. The quantitation of the reactions has been performed by unidirectional scanning of epithelia, a system successfully applied in our laboratory for the quantitation of the enzyme activity.

For doses of 4 and 8 Kr. a decrease in protein and nucleic acids concentration was obtained, and was estimated to be a logical consequence of this aqueous imbibition. However, the total quantity of those substances considerably increased. Since the increase of epithelial thickness is produced by an increased cellular volume with non-significant alterations in the number of cells, (1-3), a tentative interpretation of the results would point to a giantization of the epidermis cells not only caused by aqueous imbibition but also by an actual increase of the cellular protoplasm.

The fact that the DNA behaves differently according to the doses employed is remarkable. Total DNA quantity increases with 4 Kr and decreases with 8 Kr. This phenomenon would require an elaborate interpretation since the DNA content for a given epithelial volume is determined by the number and the activity of the nuclei, by the nuclei undergoing degenerative processes, etc.

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TABLE 1

	Proteins			Nucleic acid			DNA		
	Concentration	Total content	Thickness	Concentration	Total content	Thickness	Concentration	Total content	Thickness
Postirradiation time	r = -0.61 p 0.05	r - 0.41 p 0.05	r = 0.68 p 0.01	r = -0.80 p 0.01	r = 0.44 p 0.05	r = 0.71 p 0.01	r = -0.79 p 0.01	r = -0.63 p 0.01	r = 0.75 p 0.01
Thickness	r = -0.57 p 0.05	r = 0.83 p 0.01		r = -0.69 p 0.01	r = 0.85 p 0.01		r = -0.73 p 0.01	r = -0.31 p 0.05	

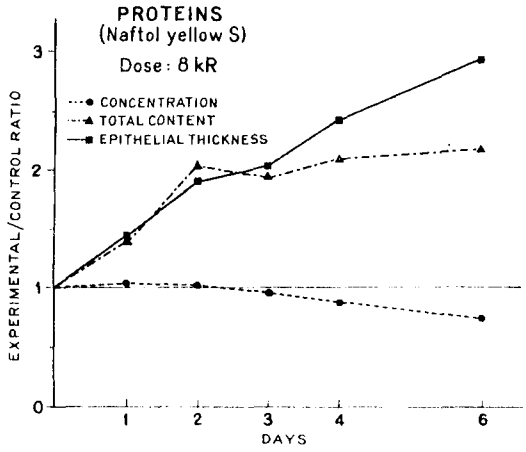


Figure 1. Microspectrophotometric determination of proteins in irradiated epidermis. Each point is the mean of four cases.

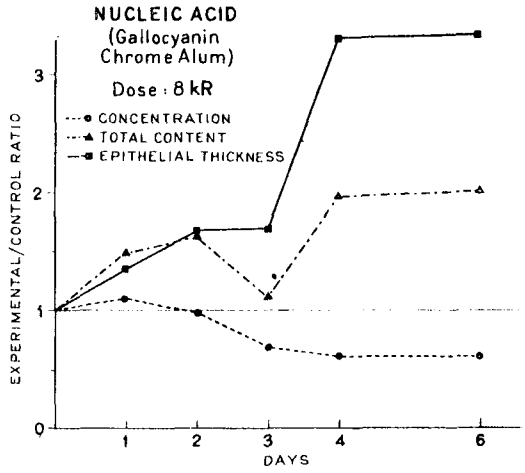
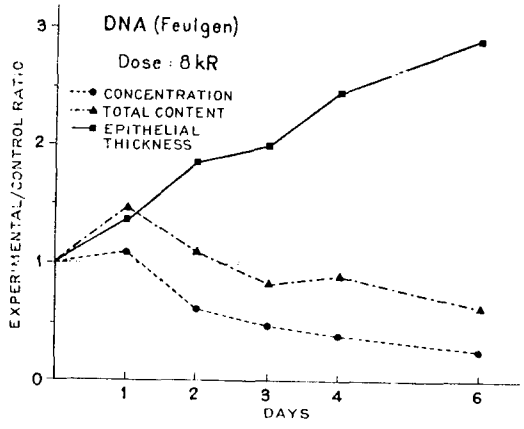
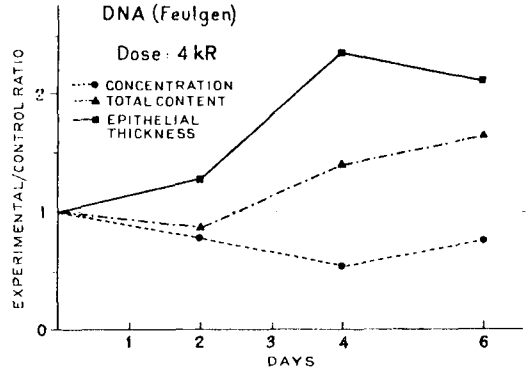


Figure 2. Microspectrophotometric determination of nucleic acids in irradiated epidermis. Each point is the mean of four cases.





Figures 3 and 4. Microspectrophotometric determination of DNA in irradiated epidermis.

F. 6 DNA Response in Epidermis Submitted to Different Doses of X-Ray radiation

C.C. Conti, I.B. Giménez and R.L. Cabrini.

One of the outstanding effects of ionizating radiation on proliferating tissues is the decrease or loss of cellular reproductive capacity. This effect, originally atributed to inhibition of DNA synthesis, seems to originate in a mitotic block process, which among others factors is probably the most important one (1).

Microspectrophotometric determination of DNA in individual nuclei, although a suitable method for studying this phenomenon, has not been employed as often as would be expected. Instead, reports in this field have dealt with neoplasic tissues (2-3).

In a previous paper we had found that DNA content of the irradiated epidermis varied according to the dose employed (4). The aim of the present study was to determine the DNA pattern of individual nuclei of irradiated epidermis in order to obtain new information about the DNA metabolism in non neoplasic irradiated cells.

2 cm areas of the mid-third of the tail of 4-5 days old Wistar rats were irradiated with 230 Kv X-rays. The proximal third properly protected by a lead shield was used as a control.

After a 2 hour fixation in Carnoy's solution, the irradiated material and the respective controls were jointly processed, following routine paraffin embedding techniques. For DNA content determinations the Feulgen staining procedure was employed using a predetermined 35' hydrolisis time at 20° in 5N HCl. Microspectrophotometric determinations were carried out in a Zeiss Cytoscan scanning microspectrophotometer.

With the data obtained, expressed in arbitrary units, frequency histograms were plotted.

Epidermis irradiated with 2 Krads exhibited an increase of DNA content in a small number of cells. This effect could be clearly seen at day 4 but at day 7 the DNA distribution seemed to return to the control patterns (Fig. 1).

The alterations of DNA patterns in the 4 Kr. specimens was more dramatic. In this case a second modal value (nearly 4n) is found as from day 4. At day 9 the diploid peak was lower than the new one (Fig. 2).

Up to day 3 the 8 Kr. pattern resembled that of the 4KR; but afterwards an important number of cells increased their DNA amount shifting the histograms towards the right and "blurring" the modal peaks (Fig. 3).

Several authors have found differences in the behaviour of nuclear DNA in mammalian tissues. Casperson in 1958 studying nuclear DNA in a X irradiated Ehrlich tumor (2) found a considerable increase in the number of cells possessing premitotic amounts of DNA.

However after Casperson's work others studies reported different results. In human uterine tumors and tissue culture strains most of the irradiated cells showed DNA values greater than the 2c range, with an important number of cells exceding 4c and even 8 c values (probably giant cells) (3-5).

On the other hand an increase of the number of cells in the diploid tetraploid range was the major alteration found in primary cultures and bone marrow cells (6).

These results are consistent with a premitotic block.

In the present work we have been able to reproduce in a normal tissue the different DNA patterns described in the bibliography by modifying the radiation dose.

The effects of radiation on cell reproduction are well known: slowing of the movement of cells through the cell cycle (especially S phase), mitotic inhibition, giant cell formation, etc. (1). As we can easily correlate some of these effects with the DNA patterns mentioned above, a tentative interpretation of our data suggests that the different levels of radiosensitivity of these parameters would cause characteristic DNA patterns for each irradiation dose under study.

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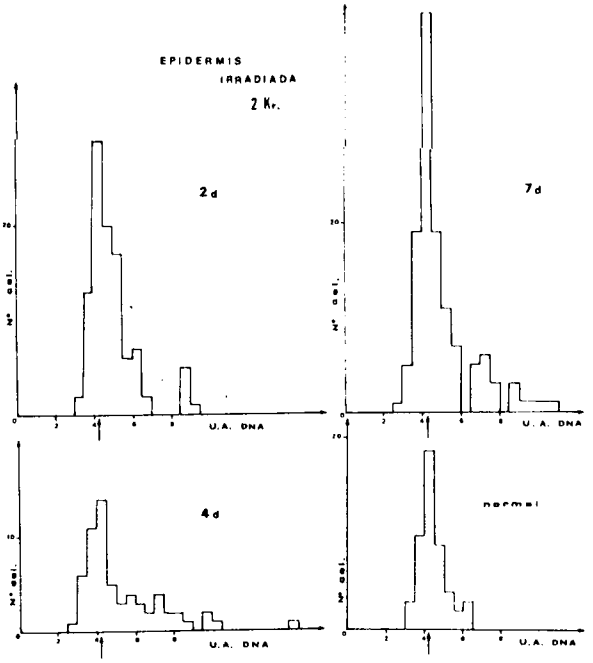

Figure 1.- Feulgen DNA histograms of epidermis irradiated with 2 Krads.

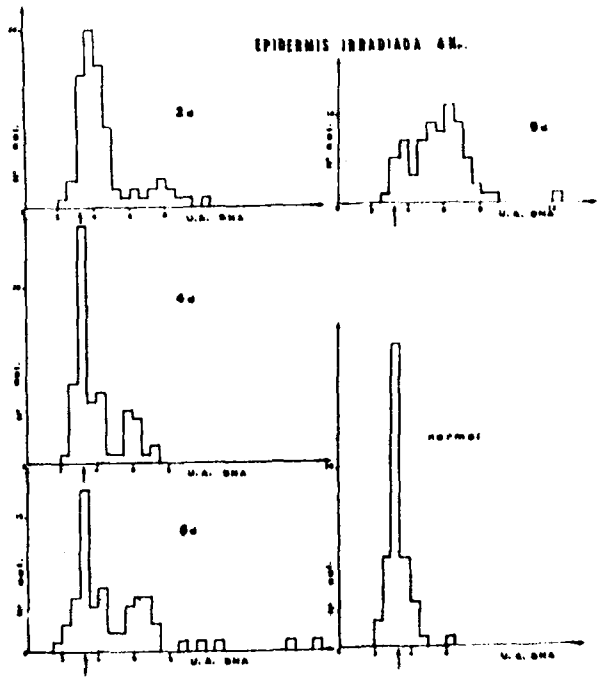

Figure 2.- Feulgen DNA histograms of epidermis irradiated with 4 Krads.

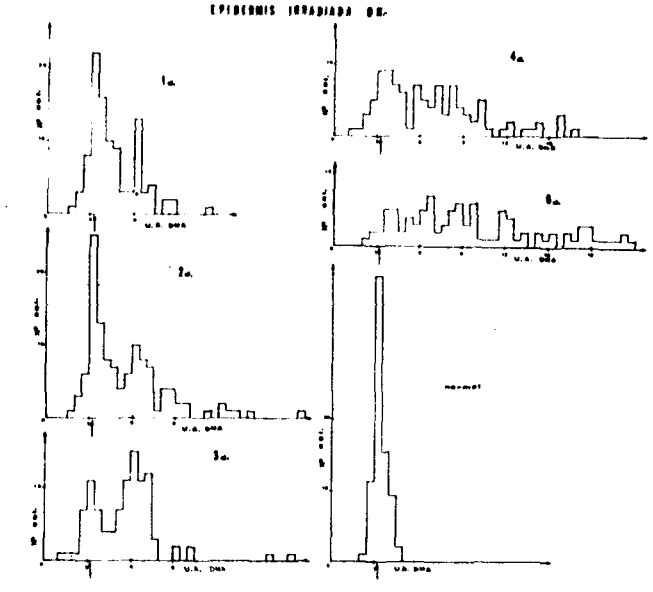

Figure 3.- Feulgen DNA histograms of epidermis irradiates with 8 Krads.

F. 7 Morphometric Determination of Volume Changes in Irradiated Keratinocytes

A.J.P. Klein-Szanto, B.M. de Rey and R.L. Cabrini.

In order to better understand the acanthogenic effect of radiation and to provide baseline data for further ultramicroscopic quantitative analysis of irradiated epidermis, a morphometric evaluation of cell volume modifications in the basal and spinous layers of irradiated and non-irradiated rat epidermis was carried out. This study was performed by applying stereologic point-counting procedures (1) which have previously proved to be an adequate and powerful method when employed in the study of squamous epithelia (2).

Two-centimeter areas of the mid-third of the tail of 4 day-old Wistar rats were locally irradiated. X-radiation was applied in the following conditions: 230 Kv, 13 mA using a 0.25 mm Cu, 1 mm Al, Filter (HVL 12.4 mm Al), dose rate 500 rads/min, total dose received 16 Krads.

Groups of five animals were sacrificed 24, 48, 72 and 92 hours after irradiation. The skin was fixed in a solution containing 2.5% gluraldehyde and 2% paraformaldehyde buffered with sodium cacodylate subdivided into several 1 mm thick blocks cut perpendicularly to the epithelial surface, post-fixed for 2 hours in 1.33% osmic acid in phosphate buffer at 4°C and embedded in Epon 812. From each animal 3 irradiated and 3 non-irradiated blocks were selected on the criterion of the best possible orientation, and 1-2 µm thick cross sections were prepared and stained with PAS and toluidine blue.

In order to obtain the absolute values of the cell volumes (V), determination of the numerical density (N_V) of both the basal and the spinous layers in the interfollicular areas of the epidermis were carried out.

The volume density (V_V) of the epidermal strata were determined in function of the epidermis as a whole, applying stereological techniques (1).

Data on the numerical density of epidermal cells, which were obtained by stereologic procedures, permitted calculation of the absolute keratinocyte volume.

The N_V of both layers under study decreased during the post-irradiation period of 96 hours. This decrease is reciprocally expressed in a marked absolute volume increase. In the basal layer the keratinocytes increased gradually, their volume reaching a maximum of approximately three times the non-irradiated volume values 96 hours after radiation, whereas the spinous keratinocytes showed a similar pattern reaching maximum values of aproximately two times the normal after the same post-irradiation period. Similar results were obtained when volumetric calculations were performed directly on one block per animal using electronmicrographs.

A one way analysis of variance of the control and irradiated samples showed that the absolute keratinocyte volume was significantly modified by radiation exposure.

A separate analysis of variance in which only the irradiated groups were contrasted demonstrated that cell volume varied as post-irradiation time elapsed.

The volume density changes of the epidermal layers showed a different variation pattern. While the V_V of the basal layer decreased approximately 20% from the control values during the first few days after irradiation, the V_V of the spinous layer increased approximately 40% during the same period (Table 2).

The volumetric changes occurring in irradiated epithelia have been previously related to a marked aqueous inhibition of the keratinocytes (3). Recent quantitative studies of enzymes, protein, nucleic acids, and sulphydryl groups (4) and stereologic analysis of epidermal organelle composition (5) have demonstrated that the lethally or sublethally irradiated keratinocyte is capable of markedly increasing its metabolic activity as well as the number and volume of important cytoplasmic organelles. These studies indicate that the hypertrophy of irradiated epidermis cells is due to both an increase in intracellular water content and an increment in the cytoplasmic as well as nuclear organelles and components.

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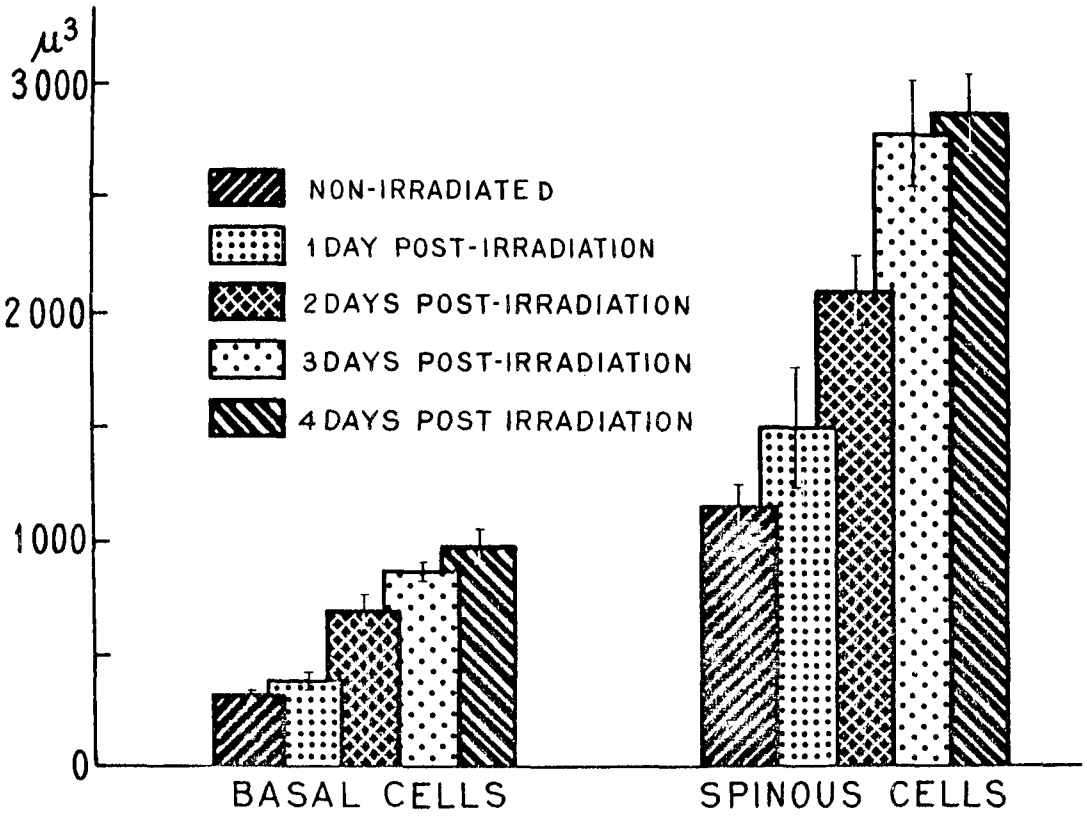

Figure 1.- Normal basal and spinous cells are compared with the 16 Krads irradiated keratinocytes. A one way analysis of variance showed a significant change in volume due to radiation exposure. For basal cells F ratio = 87 with a F.99 (35) = 3.91, and for spinous cells F ratio = 68 with a F.99 (35) 3.91. When only the irradiated groups were contrasted with this statistical test the F ratio = 29 for a F.99 (16) = 5.29 for basal cells and a F ratio = 21 for a F.99 (16) = 5.29 for spinous cells.

TABLE 1

Number of epidermal cells per 1 mm^3 of epidermal volume (N_V)

	Basal Layer	Spinous Layer
Non-irradiated	$2549 \times 10^3 \pm 1172 \times 10^3$	$560 \times 10^3 \pm 187 \times 10^3$
1 day post-irradiation	$1586 \times 10^3 \pm 656 \times 10^3$	$428 \times 10^3 \pm 57 \times 10^3$
2 day post-irradiation	$503 \times 10^3 \pm 161 \times 10^3$	$252 \times 10^3 \pm 40 \times 10^3$
3 day post-irradiation	$620 \times 10^3 \pm 351 \times 10^3$	$225 \times 10^3 \pm 49 \times 10^3$
4 day post-irradiation	$374 \times 10^3 \pm 195 \times 10^3$	$182 \times 10^3 \pm 30 \times 10^3$

TABLE 2

VOLUME DENSITY OF EPIDERMAL LAYERS IN mm^3 OF EPIDERMAL LAYER PER 1 cm^3 OF EPIDERMIS *

	BASAL LAYER**		SPINOUS LAYER**	
Non-Irradiated	204 ± 53	(n = 20)	581 ± 72	(n = 20)
1 day post-irradiation	165 ± 27	(n = 5)	642 ± 34	(n = 5)
2 day post-irradiation	171 ± 28	(n = 5)	751 ± 46	(n = 5)
3 day post-irradiation	158 ± 32	(n = 5)	735 ± 53	(n = 5)
4 day post-irradiation	168 ± 44	(n = 5)	746 ± 66	(n = 5)
F ratio ***	4,05		23,52	
F.99 (35)	3,91		3,91	

* The total epidermal volume did not include the horny layer

**mean ± standard deviation (n=number of animals)

***One way analysis of variance for irradiated and control samples

F. 8 Stereology of Irradiated Epidermis

B.M. de Rey, A.J.P. Klein-Szanto and R.L. Cabrini.*

In a previous papers we have described ultrastructural morphologic alterations of squamous epithelia submitted to high doses of ionizing radiation (1, 2). Although descriptive morphology is of primary and undisputed importance it should be complemented by quantitative techniques which yield objective and reproducible values to be contrasted by statistical tests. The aim of this research is to analyze the epithelial response to irradiation, applying stereological techniques in order to elucidate the sequence of events that characterizes the response of a heterogeneous cell populations during different keratinization stages.

The mid-third portion of the tail of new born Wistar rats was irradiated with 16 Krads as previously described (1). Sampling procedures were performed as previously described (3). Measurements were done with the aid of a coherent multipurpose grid containing 891 light points.

Point intersection, and number counting procedures were the basis for the stereological analysis (4). All data were contrated with Anovar tests.

Results for the morphometric analysis are sumarized in figures 1-7. The reference systems used were a cubic centimeter of epithelium and the absolute volume of an average basal or spinous cell. Such a design allowed to describe tissue as well as cell response to radiation injury. At the tissue level the information gathered provided evidence that 48 and 72 hours after irradiation, in a cubic centimeter of epithelium the basal cell population is reduced to a fourth, and that the spinous cell population decreases approximately two times, in comparison with the corresponding non-irradiated controls.

Furthermore this amount of experimental tissue contained fewer mitochondria and exhibited a reduction of nuclear volume and nuclear and plasma membrane surfaces. On the contrary cytoplasmic filaments were significantly increased.

Stereologic parameters expressed per absolute volume of an average cell indicated the behaviour of functional units. Although the volume of basal and spinous cells have gradually increased (5) the volume of different cell components have also reached higher values. The schematic picture of a irradiated cell could be delineated as follows: a hypertrophic cell with six-fold larger nucleus, an increased nuclear membrane surface, total number of mitochondria, double than that of non-irradiated cells, and the amount of tonofilaments four-times higher.

Traditional concepts regarding irradiated cells have lead to describe them as "edematous" cells. The present results suggest a new interpretation of irradiated cell behaviour. Thy might be considered as functional entities adapted to new physiological conditions with homeostatic mechanims operating to reach a stage of equilibrium that would account for tissue recovery. Failure of adaptative mechanisms would lead the system to a point of no-return and consequently to cell death. Declining values obtained for several parameters at 72 hours, would indicate the entrance in a degenerative phase that would result in tissue necrosis.

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* We gratefully acknowledge the collaboration and generosity of Dr. H.E. Schroeder, University of Zurich who provided the computer program for the stereological analysis of stratified epithelia.

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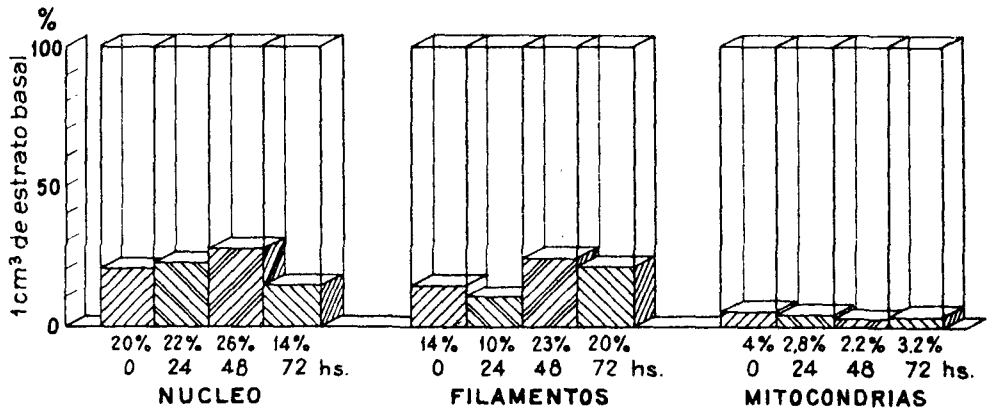


Figure 1.- Radiation induced alterations in the total volume of the nuclear, filaments and mitochondria content of 1 cm^3 of basal layer.

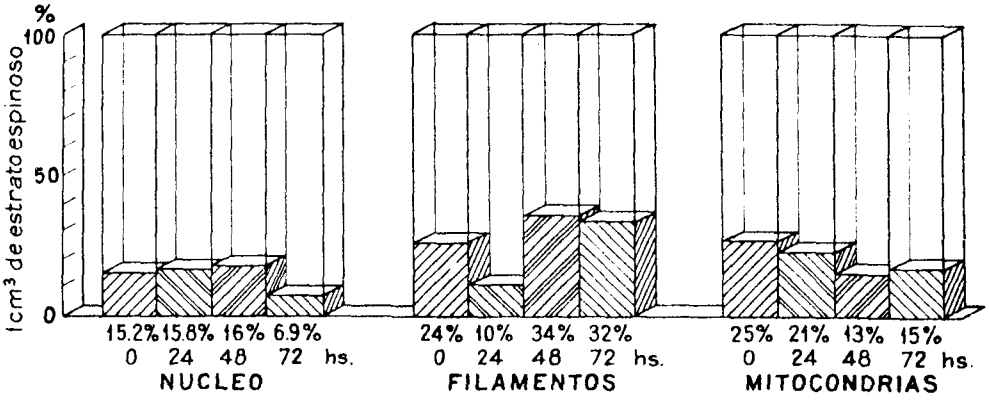
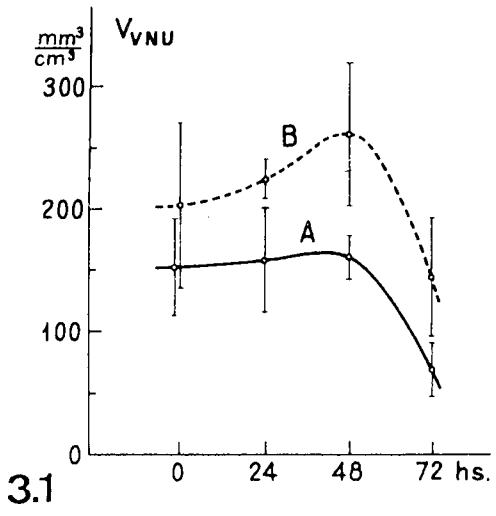
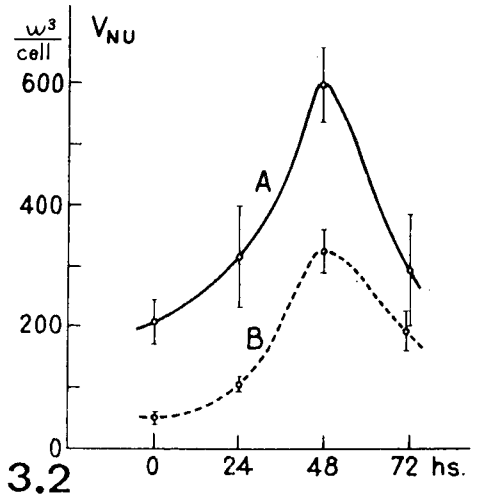
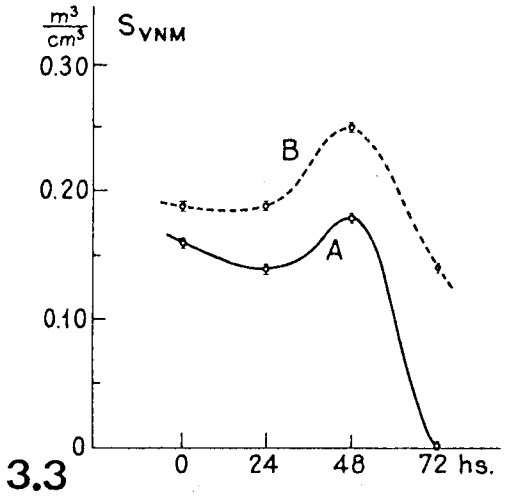


Figure 2.- Radiation induced alterations in the total volume of nuclear,filaments and mitochondria content of spinous layer.







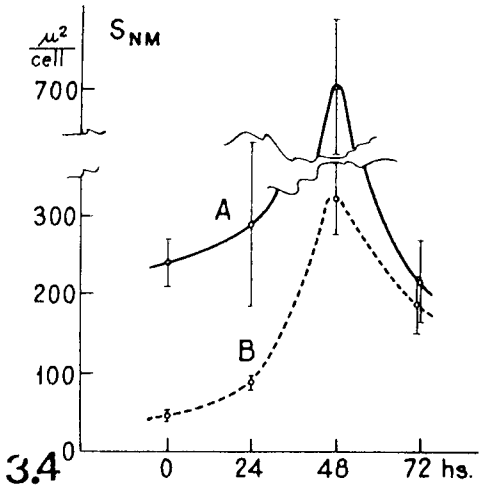


Figure 3.- Changes induced in epidermal nuclei, 0 to 72 hours after irradiation with 16 Krads of X-rays.

3.1 In volume density ($V_V$nu)

3.2 In absolute volume (Vnu)

3.3 In surface density of nuclear membrane ($S_V$nm)

3.4 In absolute surface of nuclei membrane per cell (Snm)

A: spinous layer B: basal layer

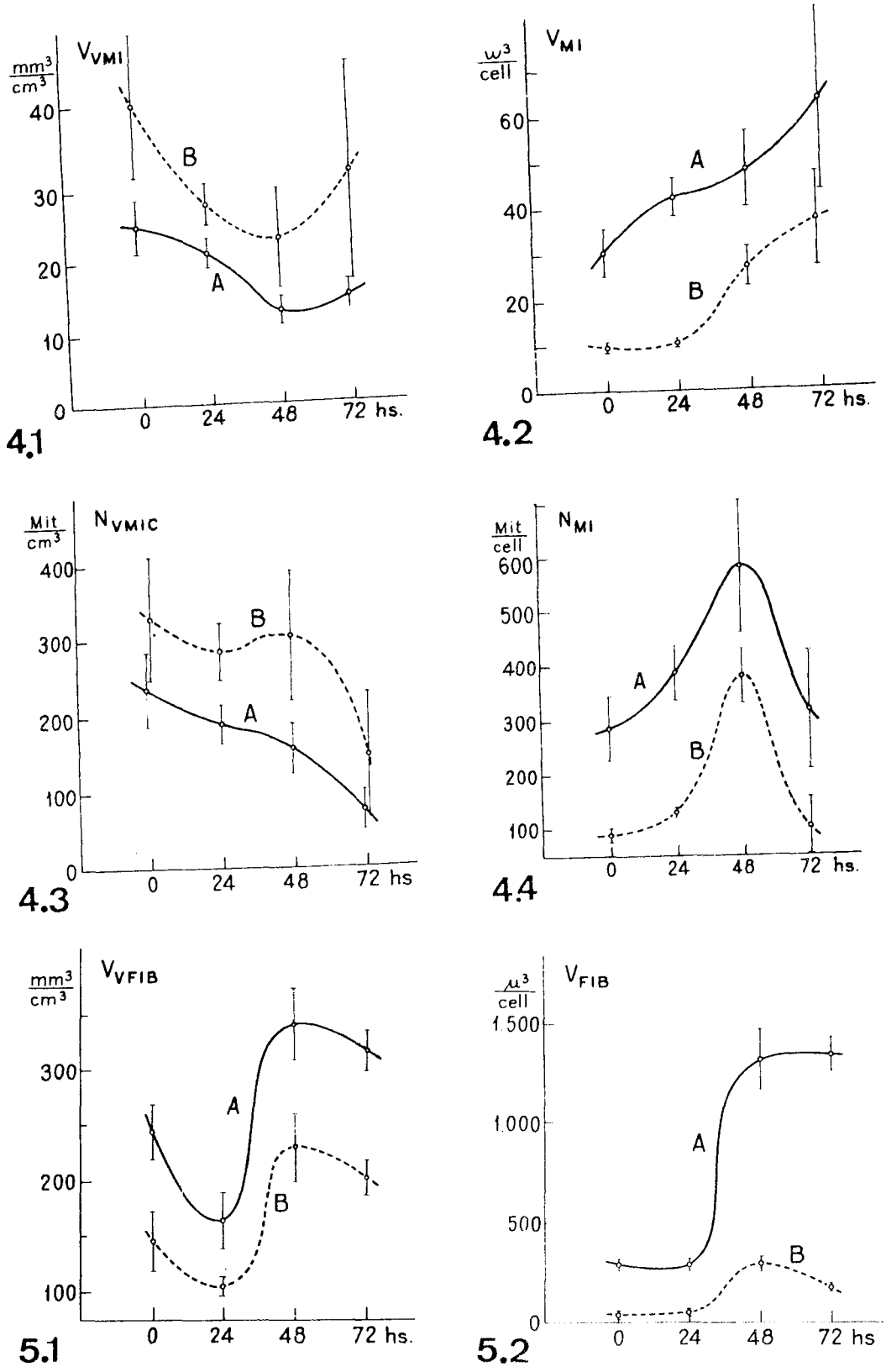


Figure 4.- Changes in mitochondrial parameters induced by 16 Krads of X rays.

4.1 In mitochondrial volume density ($V_v$mi)
4.2 In the volume of mitochondrial portion of the cell (Vmi)
4.3 In the number of mitochondria per cm^3 of epidermal strata ($N_v$mic)
4.4 In the absolute number of mitochondria per cell (Nmic)

Figure 5.- Changes in the cytoplasmic filaments.

5.1 In volume density ($V_v$fib)
5.2 In the filament volume per cell (Vfib)

A: spinous layer B: basal layer

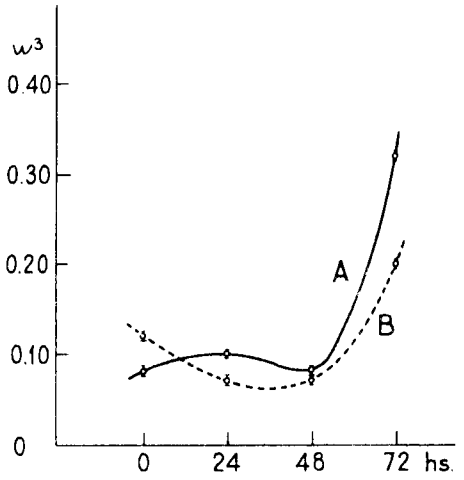


Figure 6.- Absolute volume increase of mitochondria from the basal layer (B) and spinous layer (A). The value at 72 hours are statistically significant (p 0.01).

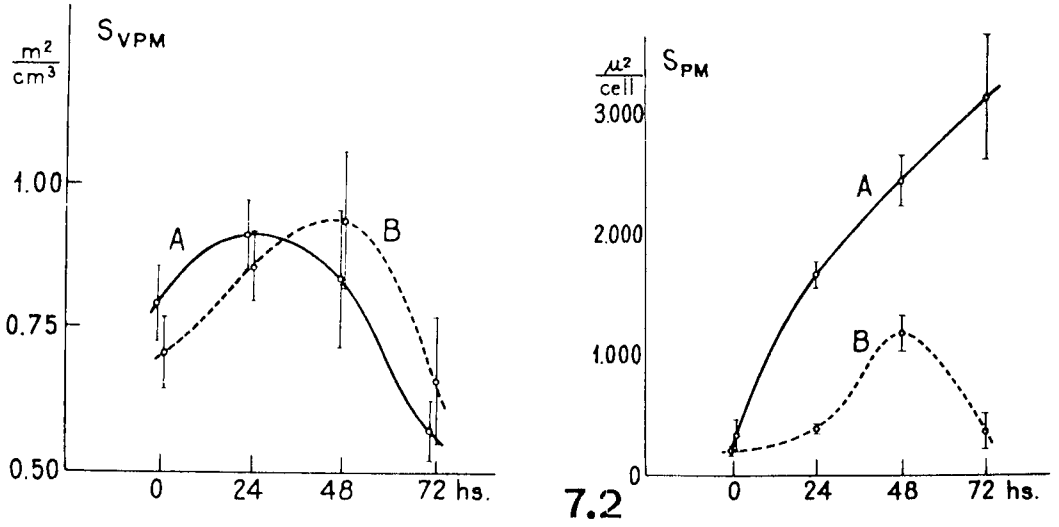


Figure 7.- Change in the plasma membrane.

7.1 In surface density ($S_v$pm)

7.2 In the surface of the plasma membrabes per cell (Spm)

A: spinous layer B: basal layer

F. 9 Effects of Local Irradiation (Head and Molars) in Rats Subjected to Orthodontic Movements

A.M. Ubios, A.C.C. Frasch, R. López Otero and R.L. Cabrini.

Bone resorption is one of the most interesting biological problems. A purely mechanical method of provoking bone resorption is the exertion of an orthodontic force, which causes resorption of the alveolar bone, primarily in the areas under pressure.

We have already demonstrated that once the orthodontic force is applied, a rapid and progressive increment in the proportion of resorptive areas occurs, while osteoclasts increase in number after an interval of several hours (1).

In whole body irradiated (WBI) animals (900 rads) subjected to the orthodontic force, we found a progressive increase in the proportion of the resorptive areas and in the number of osteoclasts, which always followed the same sequence observed in non-irradiated animals (2).

This investigation was carried out with the object of finding out the effects of local X irradiation on bone resorption under the above mentioned conditions.

Wistar rats of 200 grams weight, were irradiated as follows. Head irradiation with doses of 1,200 rads and molar irradiation with doses of 3,000, 6,000, 20,000 and 40,000 rads.

Forty eight hour after the irradiation a group of these irradiated animals was subjected to an orthodontic force during 48 hs and then killed. Another group was subjected only to 48 hs of orthodontic force, and finally, a group of animals not subjected to any experimental condition was studied.

The results were obtained by histological and histometrical methods (1).

In contrast to total body irradiation with 900 rads, local irradiation of the head with 1,200 rads, and molar irradiation with doses of 3000 and 6000 rads, did not introduce significant changes, at ninty six hours after the application of the orthodontic force, when compared with those animals only subjected to irradiation. However, when the radiation dose was increased to twenty thousand rads, the percentage of resorption, as well as the number of osteoclast, showed greater increments in bone resorption as compared to animals subjected to a nine hundred rads WBI (Figs. 1 and 2).

In animals subjected to 40,000 rads orthodontic force although exhibiting a lower percentage of bone resorption (54.12%) increased the number of osteoclast.

This data lead us to say that irradiation does not affect the mechanisms which set off the resorptive processes due to the application of an orthodontic force.

It is evident that WBI was more effective than locally employed radiation in order to bring about similar changes. This appears to be a reasonable fact, since in WBI the response is affected by a number of general factors. In consequence we have demonstrated that osteoclasts behave as in non irradiated animals.

We cannot exclude the participation of the osteoclast in the resorptive process, but whether this process is initiated by the osteoclasts or not remains unclear. On the other hand, it is evident that irradiation did not check

osteoclasis, in any of the doses employed, when the process was started applying an orthodontic force. In a previous investigation we had found that they did not form by mitotic division of the nuclei, since they did not synthetize DNA (3). Irradiation has acted over a cell formation process where mitotic division does not participate. That would explain why the number of osteoclasts can increase in such animals subjected both to irradiation and orthodontic force.

The great increase in the number of osteoclast in those animals locally irradiated with 40,000 rads and subjected to orthodontic force could be due to an increased necessity of the scavenging processes.

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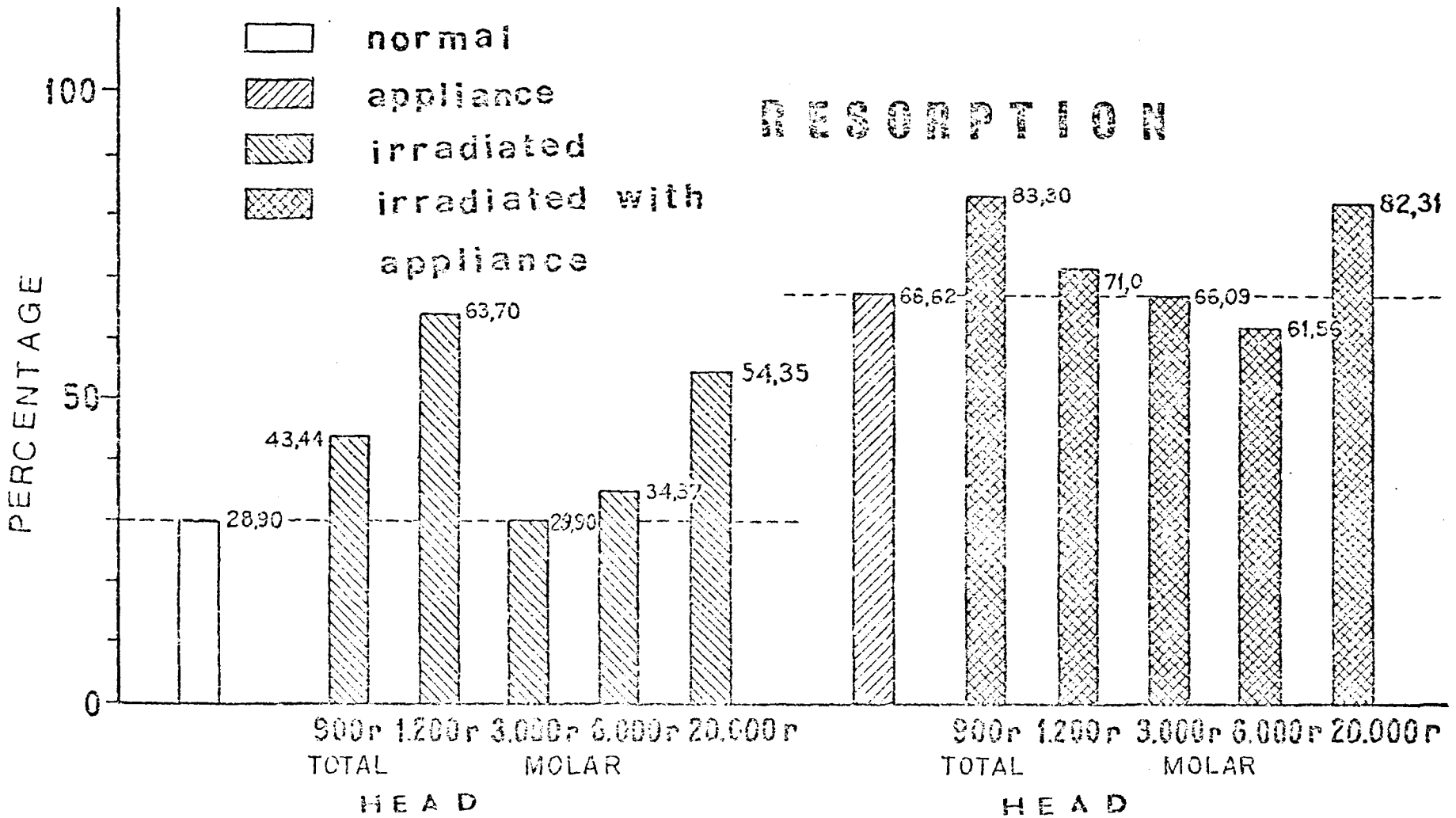

Figure 1.- Ordinates represent the percentage of the proportion of bone resorptive areas; the dotted line represents the control value. The first column indicates the values obtained by the application of the orthodontic force for 48 hours.
The values obtained with the different irradiation conditions with and without appliance are shown in the rest of the columns.
The difference was significant for WBI, but not for local irradiation on head and molars with 3,000 and 6,000 rads, between animals irradiated only and those irradiated and subjected to the orthodontic force. But with 20,000 rads, we obtained similar values to those of nine hundred rads WBI.

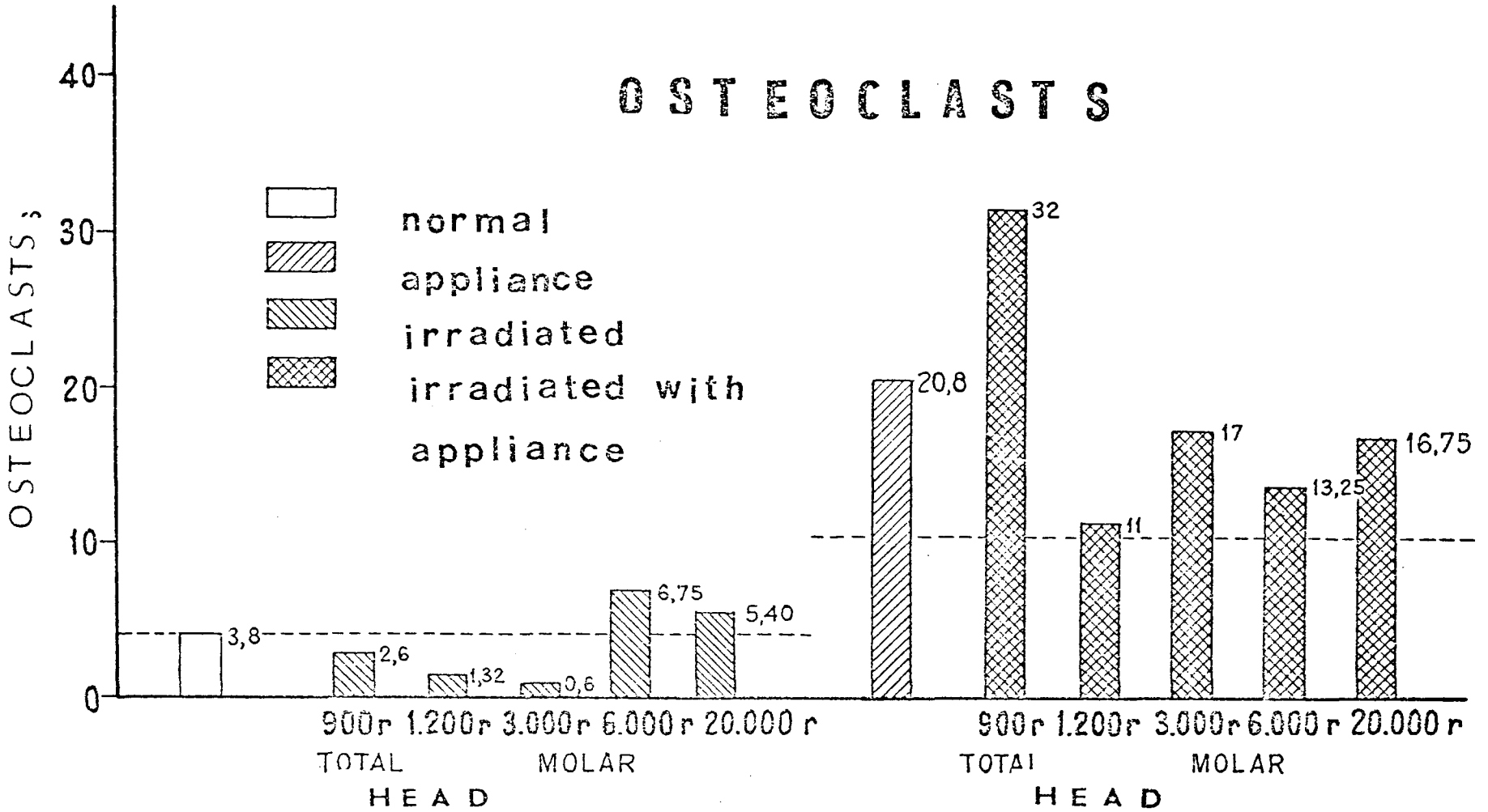

Figure 2.- As far as osteoclast are concerned no significative changes were found in those animals subjected to irradiation. But a notable increase was found in those animals subjected to WBI and force aplication.. In contrast no significant changes were found in the animals subjected to local irradiation, with or without appliance. Even with 20,000 rads, the number of osteoclasts showed a greater increase, but not as high as that of animals subjected to WBI.

F. 10 Morphometric Analysis of the Vermilion Border and Skin Epithelium

H. Lanfranchi and B.M. de Rey.

The vermilion border placed between the skin and the lip mucosa offers peculiar features and its epithelium can be considered as a transitional tissue, presenting characteristics of both the epidermis and the mucosa.

Morphometric techniques have been used by several authors (1, 2, 3) to describe precisely the oral mucosa in different areas. Nevertheless no quantitative data on the vermilion border is available. Stereologic technique were applied to different mammalian epithelia.

These techniques made it possible to compare the epithelium of the vermilion border of three mammals in which the morphologic appearance was somewhat different.

10 pieces of lips and adjacent skin of cattle, Wistar rats and human biopsies were simultaneously fixed in formol and processed to be observed with the ligth microscope. The stereologic parameter used was the volume density (Vv) and all the measurements were performed with the aid of a 210 point grid. The volume density of the tissue could be determined by superimposing a systematic set of test points over the area under study. Then the test points falling over the different strata were divided by the total points of the grid. It was possible to calculate the volume of a cube (face surface equal to the grid's surface) and to relate it to the Vv values obtained for the different epidermal layers in order to calculate the absolute volume of each stratum contained in the cube's volume. Similar simple calculations were made to estimate the thickness of each layer and of the whole epidermis. Table I shows the relative volumes of the epidermal strata from vermilion border and skin. It is noticeable that values for the epidermal strata of cows are larger than those for humans or rats.

Table II shows that vermilion borders of cows are approximately 1 mm thick, and that those of rats are 100 µ. The corresponding values for skin are consistent with those for vermilion border.

In Table III shows the percentage of each strata related to the total epithelium. It is evident that values of vermilion border epithelial strata are very similar independently of the species studies.

Present data suggests that the epidermal parameters analyzed in relation to an ideal skin cube, i.e. volume and thickness of the vermilion border, are twice as large as those of skin. This increase is based upon the significantly thicker spinous layer of the vermilion border. This relative physiological increment would indicate a functional specialization of this tissue and is considered an important feature of the vermilion border epithelium. Note that in spite of the significant differences found in thickness and volume of the vermilion border for each of the species analyzed, the precentages of the different layers were almost constant in the three cases. The action of regulatory mechanisms would explain this interesting feature.

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TABLE I

Volume density of epithelial layers

Layers	Parameter	Units	Vermilion border*			Skin*		
			Cattle	Rat	Human	Cattle	Rat	Human
Horny	V_v	$\frac{mm^3}{cm^3}$	93±14	14±2	16±3	60±22	14±3	16±4
Granular	V_v	$\frac{mm^3}{cm^3}$	-	7±10	-	57±14	4±1	8±2
Spinous	V_v	$\frac{mm^3}{cm^3}$	527±6	44±11	91±8	222±47	14±5	30±9
Basal	V_v	$\frac{mm^3}{cm^3}$	54±7	7±2	11±3	47±10	6±2	10±0

* A one way analysis of variance showed a statistically significant difference between layers ($p < 99\%$)

TABLE II

Layers	Parameter	Units	Vermilion border*			Skin*		
			Cattle	Rat	Human	Cattle	Rat	Human
Horny	THICKNESS	mm	0.122 ± 0.018	0.019 ± 0.002	0.020 ± 0.003	0.077 ± 0.028	0.019 ± 0.005	0.021 ± 0.009
Granular	THICKNESS	mm		0.001 ± 0.001		0.075 ± 0.019	0.005 ± 0.000	0.005 ± 0.001
Spinous	THICKNESS	mm	0.687 ± 0.098	0.066 ± 0.026	0.121 ± 0.005	0.289 ± 0.062	0.019 ± 0.004	0.039 ± 0.011
Basal	THICKNESS	mm	0.071 ± 0.009	0.010 ± 0.001	0.039 ± 0.011	0.061 ± 0.013	0.008 ± 0.001	0.013 ± 0.005

* See Table I

TABLE III

Volume percentage occupied by epithelial layers

Layer	Parameter	Vermilion border			Skin*		
		Cattle	Rat	Human	Cattle	Rat	Human
Cornea	%	14 ±2	19 ±1	13 ±1	16 ±5	36 ±8	25 ±1
Granular	%		11 ±1		15 ±2	11 ±1	11 ±1
Spinous	%	78 ±3	59 ±10	78 ±2	58 ±5	37 ±8	48 ±2
Basal	%	8 ±1	11 ±1	9 ±1	11 ±2	16 ±1	16 ±4

* See * Table I

F. 11 Morphometric Baseline Data of Normal Human Interfollicular Epidermis

A.J.P. Klein-Szanto.*

Variations in the relative composition of keratinocyte components in experimental and spontaneous pathologic conditions have frequently been reported on the basis of subjective evaluation of electron micrographs. By this procedure quantitative changes in the cytoplamic content of tonofilaments, mitochondria, keratinosomes, and other organelles have been postulated in as different conditions as lichen planus, psoriasis vulgaris, horny layer stripped epidermis, irradiated epidermis, and many other lesions.

This study represents an attempt to describe in a quantitative and objective way the ultramorphologic features of human epidermis, in order to furnish baseline data for the study of epidermal morphometric pathology. The study was developed and carried out taking into account the previous experience in the sterologic assessment of normal squamous epithelia of the oral mucosa (1-3).

Nine skin surgical biopsies, 4 from the submammary region and 5 from the iliac crest region, were obtained from female patients age ranging from 19 to 23 years undergoing plastic surgery. The biopsies were immediatelly placed in chilled half-strength Karnovsky fixative and subdivided into 5 to 7 approximately 1 mm thick blocks, cut perpendicularly to the surface.

After 2 hr the blocks were washed in 0.18 M cacodylate buffer and postfixed in 1.33% osmic acid buffered (pH 7.4) in 0.067 M s-collidine at 4°C, thereafter dehydrated in ethanol, and embedded in Epon. The tissue blocks were orientated flat at the bottom of reversed BEEM capsules. From a total of 47 blocks, 1-µm thick sections were prepared and stained with PAS toluidine blue-azure II. From these blocks, 3 out of each biopsy were selected on the criteria of the best tissue orientation and preservation of the interfollicular epidermis.

Ultrathin sections were cut and processed according to a previously published sequence (3).

Sampling of fields was performed according to an already described model, which was performed on two levels of magnification and four epidermal strata.

In the submammary epidermis the four strata represented an almost consecutive field-to-field sample at the lower level of magnification. In the iliac crest epidermis, which was slightly thicker, small areas between the strata were equidistantly excluded from the stereologic analysis.

Level I magnifications: Six electron micrographs at a primary magnification of 3070x were recorded in each stratum and for each block. Thus three times 24 photos were colected per block, yielding a total of 288 negatives for the submammary epidermis group (4 biopsies x 3 blocks x 4 strata x 6 micrographs) and 360 electron micrographs for the iliac crest epidermis group (5 biopsies x 3 blocks x 4 strata x 6 micrographs).

Level II magnification: Tweelve micrographs were recorded using a primary magnification of 6070 x within the strata sampled at level I. In total, 40 electron micrographs were sampled from each block, yielding a total of 576 negatives for the submammary epidermis (4 biopsies x 3 blocks x 4 strata x 12 micrographs) and 720 negatives for the illiac crest epidermis (5 biopsies x 3 blocks x 4 strata x 12 micrographs).

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* This study was carried out in part at the Department of Oral Structural Biology of the University of Zurich, Switzerland.

Two additional series of electron micrographs were recorded for the measurement of cytoplasmic filament diameter and basal lámina thickness.

The quantitative analysis was carried out using a coherent double-lattice test system with 99 heavy points and 891 light points respectively.

The interfollicular epidermis of the submammary region was found to be of rather similar thickness and structure as the one of the iliac crest region although the latter was slightly thicker and presented more epithelial ridges interdigited with a somewhat denser dermal papillary body, the overall architecture, differentiation pattern and horny layer formation were similar in both regions.

The basal complex beneath the epithelium was composed of a very distinct lamina lucida and a lamina densa of approximately equal thickness in both skin rerions under study (lamina densa 510 to 580 Å; lamina lucida 370 to 390 Å).

In order to eliminate the variations in strata composition caused by the heterogeneous distribution of the intercellular space, nonkeratinocytes, and nuclear volume fractions, and to compare the data of epidermis at the two different sites, the results were expressed in relation to one unit volume of cytoplasm (Figure 1).

The nutochondrial, ribosomal, and endoplasmic reticulum volume fractions showed a marked decrease from the basal to the granular layer. Slightly decreasing gradients could be observed in the volume densities of the Golgi apparatus and cytoplasmic vesicles. The volume fraction occupied by bundled filaments increased from the basal to the upper spinous layer, exhibiting a relative decrease in the granular layer.Non bundled cytoplasmic filaments were present in the basal layer only, accounting for 0.7% and 1.5% of the cytoplasmic volume in the submammary and iliac crest epidermis, respectively. The diameter of single filaments ranged in all layers from 72 to 82 Å.

The volume density of the membrane-coating granules increased gradually up to the lower spinous layer and markedly throughout the two remaining superficial strata. Keratohyalin was present in the granular layer only, representing 5% of the cytoplasmic volume. The volume fraction of the cytoplasmic ground substance remained relatively constant throughout the four layer.

Differences in volume densities of cytoplasmic components between the two epidermal localizations were insignificant.

The essential findings on the present stereologic analysis are in general agreement with previous qualitative studies, although in applying techniques of stereologic cytology, clear cut numerical definitions of tissue and cell composition and of epithelial differentitation patterns are objectively obtained.

The stereologic study of epidermis in other regions of the human body with different structural and differentiation patterns as well as an analysis of the normal sex and age variations seems desiderable before analyzing pathologic epithelia. In this way it would be possible to obtain valuable data on different diseases as has been the case while analysing other tissues.

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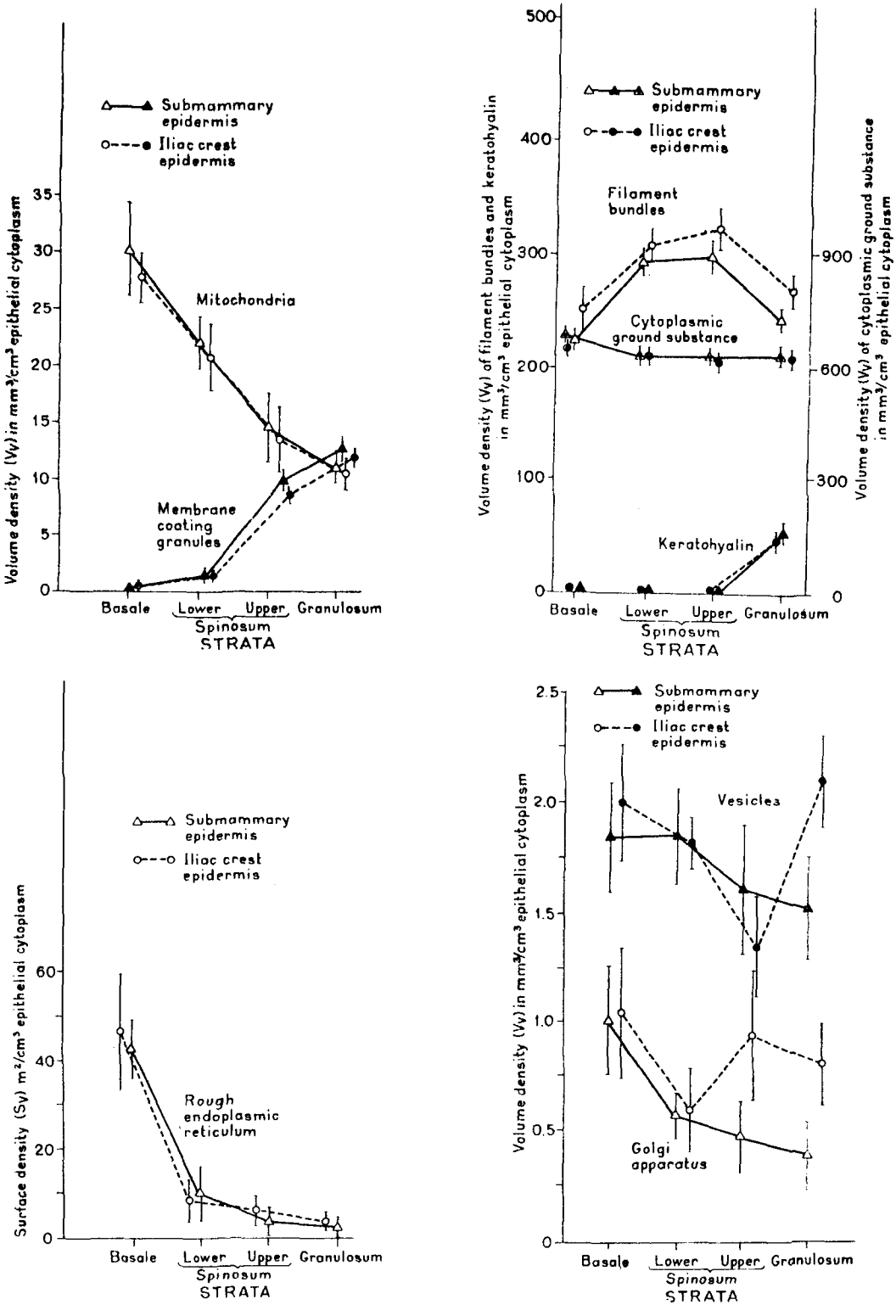

Figure 1.- Volume densities of the most important epidermal cell parameters.

F. 12 Stereology of Dark and Clear Keratinocytes in Human Epidermis

A.J.P. Klein-Szanto*

Epidermal basal cells have been classically described by light-and electron-microscopy as forming a homogeneous population of cuboidal or columnar cells. The description of dark and clear epithelial cells in normal and pathologic epithelia and especially the occurrence of these two cell-type in normal epidermis (1) indicate the presence of morphologically different keratinocytes in the stratum basale. Furthermore, studies of epidermal cell kinetics have shown that at least from this point of view, the epithelial basal cell population is composed of functionally different cells.

The aim of this study was to determine the proportion of dark and clear basal cells in normal human epidermis of the iliac crest region, and to analyze the ultramorphology of these cells in comparison with keratinocytes of the lower spinous layer, using techniques of stereologic cytology.

Surgical skin biopsies from the iliac crest were harvested from five caucasian female patients (age range 19 to 22 years) undergoing plastic surgery. The biopsies were processed for light-and electron-microscopic observations using previously described techniques (2-3). From each biopsy one block containing dark and clear epithelial basal cells was selected on the criteria of best possible tissue orientation and preservation.

Electron micrographs were recorded following an already described sampling design based on a model of stratification. It was performed on two levels of magnification and applied to a) dark epithelial basal cells, b) clear epithelial basal cells, and c) suprabasal cells of the lower spinous layer.

Level I: Ten electron micrographs per block at a primary magnification of x 3070 were recorded in each of the three different cell populations (total of 150 electron micrographs).

Level II: Twenty electron micrographs were recorded using a primary magnification of x 6070 within the fields and cell populations sampled in level I (total = 300 electron micrographs).

The quantitative analysis was carried out by applying stereologic procedures using a coherent double lattice test system for stereologic cytology with 99 heavy points and 891 light points (4).

The suprabasal layer was composed of a rather homogeneous epithelial cell population of polyhedral cells which amounted to 98.80% of the stratum's volume. Non-keratinocytes represented 1% of the total volume, whereas the intercellular space accounted for less than 1 o/oo of the volume of the suprabasal layer.

In the basal layer dark epithelial cells represented on the average 33% of the total volume, clear epithelial cells 65%, non-keratinocytes 3.40% and the intercellular space 1.70 o/oo (Table).

Data calculated per cell are summarized in the Figure.

Dark basal cell contained significantly higher amounts of melanin-containing organelles and bundled filaments than the clear epithelial cells ($p > 0.05$ using Student's test for paired data), whereas these cells were characterized by the presence of higher volume fractions of non-bundled filaments and cytoplasmic ground substance ($p > 0.02$ using Student's test for paired data).

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* This study was carried out in part at the Department of Oral Structural Biology of the University of Zurich, Switzerland.

In comparison with basal cells, the suprabasal cells contained lower volume fractions of mitochondria, rough endoplasmic reticulum, Golgi apparatus, non-bundle filaments, and melanin-containing organelles. Desmosomes found in relation with dark basal keratinocytes were more abundant than those associating the other cell types studies,

The quantitative evaluation of several parameters revealed differences in many important contituentes of these cell types. In comparison with the clear basal cells, dark cells were characterized by a higher surface density of bundled filaments and melanin-containing organelles, a diminished volume fraction of the cytoplasmic ground substance and a larger number of desmosomes.

Although many parameters showed differences with their suprabasal counterparts, some of these features were similar to those observed in suprabasal cells. Notwithstanding this fact, the tendency of these results seems to indicate that intermediate values between those characterizing clear epithelial basal cells and those of suprabasal keratinocytes, frequently fit the obtained numerical pattern of dark cells morphometric composition.

Dark basal cells characterized by a high content of bundled filaments and melanosomes could easily be regarded as members of a second basal cell sub-population undergoing a process of differentitation while remaining in the basal layer. Furthermore radiobiological and carcinogenesis studies suggest the existence of basal cell subpopulations which react in different ways to the used experimental stimuli (5-6).

In addition, the presence of an irregular and folded nuclear membrane and membrane-bound organelles, revealed by the increased surface density of these cell components, is in accordance with previously described characteristics of aging cells.

In spite ot the fact that these features of dark basal keratinocytes may also be interpreted as degenerative (Breathnach, 1971), the dark basal epithelial cells may represent differentiating keratinocytes which either remain for an undefinable period of time in the stratum basale or are actually beginning migration towards the suprabasal layer.

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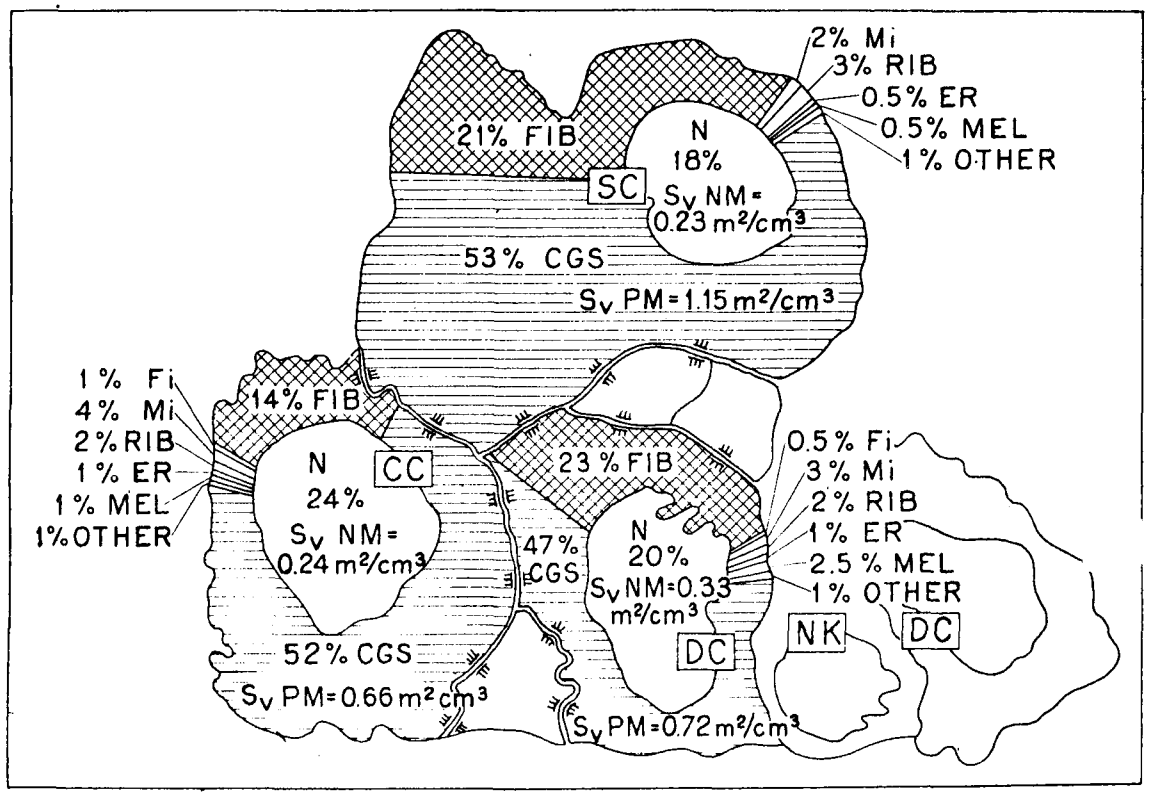

Figure 1.- Volume fractions of cell components expressed in percentage of each cell's total. Volume - SC: suprabasal cell; C: clear basal cell; DC: dark basal cell; NK: non keratinocyte; N: nucleus; Fi: cytoplasmic filaments; Fib: filament bundles; Mi: mitochondria; Rib: ribosomes; ER: endoplasmic reticulum; M: melanosomes; SGS: cytoplasmic ground substance; S_V NM: surface density of the nuclear membrane; S_V PM: surface density of the plasma membrane.

F. 13 Ultrastructural Response of Epidermis to Actinomycin D.

B.M. de Rey and R.L. Cabrini.

In previous papers we analyzed the behaviour of rat epidermis submitted to ionizing radiation using ultrastructural and histochemical methods (1). A series serie of alterations induced by different dose levels cytoplasmic and nuclear of alterations induced by different dose levels affects cytoplasmic and nuclear zation mechanism. To analyze the intimate causes of these processes, we have used drugs of perfectly known action at molecular level. 0.04 ml of actinomycin D was subcutaneously injected at the rate of 200 µg/kg of animal weight and the area of injury was marked, 40 h after injection skin samples were obtained from this area and from other normal ones.

The basal and spinous cells show an increased degree of intercellular edema (fig. 1) not observable among the granular and horny cells. Acanthosis is slight for the whole epidermis, no changes are observed in the dermoepidermal junction. Clear cells, alternate with others of greater electron density. The former show a light background with scarce ribosomes while electron dense cells show large aggregates of perinuclear glycogen and numerous ribosomes.

Abundant altered mitochondria, with disrupted membranes and disorganized crests are found in numerous cells. Groups of large electron dense lipid vacuoles are also seen in close contact with the perinuclear mitochondrial groups. They are seldom found scattered through the cytoplasm. Tonofilament bundles are reduced in size in most basal and spinous cells and they sorround the nuclei. The nuclei show increased volume, unaltered membranes, neither fragmentation nor nuclear lobulation and scarce nuclear inclusions. The most outstanding feature of drug action is the nucleolar alteration observed in all the epithelial layers.

The segregation of the nucleolus components, shows different morphological features depending on the epithelial layer. It is in the upper spinous layer and in the granular layer that the images of a total segregation between the nucleolar elements (fibrilar and granular areas) can be observed.

The study at ultrastructural level of the effects of actinomycin D on epidermis enables us to morphologically characterize a well-known process at molecular level. This inhibitory drug inactivates the DNA templates involved in the synthesis of ribosomal precursor (2).

In the present study important subcellular alterations affect mitochondria and endoplasmic reticulum and their morphological changes resemble those induced by ionizing radiation (1-3).

Perinuclear lipid vacuoles, a feature of irradiated skin, also appear in actinomycin D treated skin. The presence of clear cells with low density of ribosomes and tonofibrils would reflect at cytoplasmic level the molecular alterations experienced by the precursors ob ribosomal RNA. The nuclei, with the exception of nucleolar alterations and increased size, do not offer major changes. This would imply a marked difference with respect to the nuclear lesions induced by ionizing radiation, which brings about lobulated and fragmental nuclei. The important alterations, of all cellular layers may be considered as ultrastructural reflection of molecular dysfunction.

It is interesting to point out the changing morphological appearance of the altered nucleoli in connection with the epithelial layers which would follow a chronological pattern similar to that described by Simard and Bernhard (4). In

the basal and proximal spinous layers the nucleolar segregation would fall within the definition of microsegregation (5) in which dense and lighter areas of fibrillar and granular components are identified.

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F. 14 Thyamine Pyrophosphatase and Acid Phosphatase. Their Ultrastructural Localization in Rat Skin*

M.E. Itoiz, B.M. de Rey and R.L. Cabrini.

Localization in normal rat skin of the thyamine pyrophosphatase enzyme (TPPase) which catalyzed cocarboxylase hydrolysis, is analyzed in this paper. The activity of this enzyme has been detected at ultrastructural level in other tissues (1) but it has not been described for normal skin structure. An effort has also been made to correlate TPPase existence with AcPase, at epidermal surface layers.

Skin specimens 1 x 2 mm from tails of 10-15 days-old Wistar rats were fixed in 2% cold glutaraldehyde in cacodylate buffer for a period of 15 min, were cut and then incubated for TTPase demonstration, according to the technique reported by Goldfischer et al (2). For AcPase demonstration, Gomori's technique, modified by Bark (3), was followed.

Control sections,from rat liver and kidney, were incubated together with rat skin sections. Other control skin sections were incubated without substrate or by adding 0.02 m FlNa for AcPase inactivation.

After incubation, sections were washed in buffer, post-fixed in 2% osmium, dehydrated and embedded in Epon 812. Inclusion was accomplished following a technique that enables ultrathin oriented sections to be obtained from the 30 μ thickness of the frozen sections.

Ultrathin sections were observed in a Philips 300 electron microscope.

TPPase appeared localized both in dermic and epidermic structures. An intense reaction of the pinocytic vesicles of the vascular endothelium was observed. A similar intense reaction was also detected in the Golgi apparatus of endotelial celss and fibroblasts. In most of the cells of the stratum basale spinosum and granulosum, the only observable reaction corresponded to the Golgi apparatus. Such activity was detected in the outer saccules of the dictyosome leaving a reaction-free central portion which correspond to the vesicles.

In the horny layer, mostly in intercellular spaces, intensely reactive bodies have been observed, of approximately 100 300 A diameter. In some of these bodies laminar striations under the reaction product were observable.

Some striated or membranous elements with a diameter which may reach 500 A,were also observable within the horny cells.

In the sections FlNa inactivated all the described elements.

AcPhase, appeared to be active in all the epithelial cells. A small number of reactive lysosomes were manifest within basal cells, keratinosomes seemed more abundant in the malpighian granular cells, where they were found localized close to the cell membrane.

Many striated granules were seen in intercellular spaces of granular and horny layers. In the granulous cells an intense and diffuse precipitation was observed.

It was striking to find horny cells with an intense diffuse reaction, surrounded by other reaction freecells.

The membranous elements within the horny layers, found to be reactive toward TPPase, also displayed AcPase activity.

The controls incubated with FlNa showed total inactivation of AcPase positive elements.

Acid phosphatase activity patterns found in rat skin are, in general, similar to those already described for human skin or for other laboratory animals (4, 5).

The localization of TPPase activity in the Golgi apparatus of dermic and epidermic cells is also similar to that described for other tissues. The repeatedly suggested usefulness of this reaction as marker of the Golgi apparatus becomes particularly relevant at epidermal level, bearing in mind the difficulties involved in localizing it in routine preparations.

The appearance of TPPase and AcPase positive elements on superficial layers, which can be identified as keratinosomes or as a discharge product of this organelle in the intercellular space, is an interesting finding.

It is noteworthy that in the spinous cells there are no positive TPPase granules, while positive AcPase keratinosomes are observed.

The persistence of TPPase active granules in FlNa incubated sections and their total inactivation in the case of incubations for AcPase tend to suggest a coexistence of both active enzymes in the surface layers keratinosomes.

Hashimoto (6) has proved the existence of phospholipids within these granules, which he called "cementosomes". Together with the presence of acid phosphatase and ATPase, the existence of these phospholipids would bring to presume that these granules are not merely "membrane coated structures containing acid phosphatase", but more complex elements. Different substances are found to coexist within them, and several enzymatic activities would arise in the course of cellular differentiation stages.

It is a controversial point, still subject to further elucidation, whether the keratinosomes are involved in the cellular death process as in scaling, or in the formation of an intercellular cementing substance of the most superficial epidermal cells.

The largely reactive zones within the horny cells would apparently correspond to infolded plasma membranes.

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F. 15 Adaptive Differences of the Surface Organization of Horny Cells.

B.M. de Rey and C.J. Conti.

The aim of the present study was to analyze the surface architecture of both the upper and lower cell surfaces of the stratum corneum of keratinized epithelia of rats and humans in several regions of the body and at different ages, in order to find a possible relationship between the structural organization on cell surfaces and the prevailing enviromental mechanical stimuli.

Pieces of skin from abdomens, palms, legs and soles from 5, 20 and 75 day-old Wistar rats were processed in order to observe the upper and lower surfaces of corneocytes with the Scanning electron microscope (SEM).

The horny cell surfaces of the epidermis of palms and soles of Wistar rats, 16 to 35 days old, showed peculiar structural patterns. These structures differed morphologically in the lower as well as in the upper surface of the horny cell. The lower surface displayed abundant well developed villi of a mean diameter of 1.5 x 0.5 µm. The upper surface of the horny cells revealed pores of approximately 1 µm diameter. Both villi and pores seemed to be better developed and to have a more regular shape in deeper regions of the horny layer. The corneocytes of palmar and plantar epidermis of new-born rats did not reveal this type of surface structure, although a finely folded reticular pattern could be noted.

SEM observations in humans showed a similar distribution of these horny cell structures in palms and soles, disclosing age-dependent variations.

The stratum corneum as a whole was remarkably resistant to distruption by mechanical means (1), yet the superficial layers are regularly and easily shed. In case of a very strong mechanical erosion, as can happen in palms and soles, the cementing substances, apparently mucopolysaccharides (2) would not suffice to avoid an excessive desquamation.

The villus-like projections and the pore-like depressions dercribed in the present study are interconnecting and interdigitating structures of the superposing horny cell layers, which would contribute to the adhesion of the thick horny layers of palm and sole epidermis. These structures could develop in the regions highly exposed to erosion when the test animals start to move around in the cage. In humans the equivalent time is to be counted as from the moment they start to walk and grasp.

Our results seem to indicate that the cytoarchitecture found on the surface of horny cells of palms and soles in rats and humans constitute acquired adaptive structures of the epidermis which show regional age-dependent or friction-dependent variations (3).

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F. 16 The Effect of Decalcification on the Microspectrophotometric Determination of DNA

I.B. Gimênez, C.J. Conti and R.L. Cabrini.

The conventional dethods of decalcification (solubilization of Ca^{++} in acid medium) exert an active depolimerizing action on nucleic acids. This phenomenon is a serious handicap for carrying out microspectrophotometric studies on the DNA content in calcified tissues.

In our investigation we have used different decalcifying agents so as to evaluate theis use in microspectrophotometric studies of DNA in calcified tissues. Liver, spleen and the maxillae of adult Wistar rats were exposed to the action of nitric acid, picric acid, and EDTA.

The tissues were subjected to the action of 7% nitric acid for 9, 12, 24 hours and 3 days. In picric acid (Bouin Fixative) the exposure lasted 3 days and in EDTA tris pH 7, 15 days. The Feulgen reaction was carried ou using hydrolysis periods of different duration (1-2). Microspectrophotometric readings were perdormed in a Zeiss Cytoscan with automatic stage under conditions described in a previous paper (3).

The tissues treated with nitric acid and picric acid showed an important loss of nuclear DNA content. In most cases the Feulgen reaction gave a completely negative response.

The material exposed to the action of EDTA tris pH 7 yielded hydrolysis curves similar to the controls (diagram 1); the ploidy relationship of the studied tissues was not affected (tables I and II).

Our results obtained in the cells of different tissues apparently indicate that the stechiometric relation of Feulgen's reaction is not modified by treatment with EDTA tris pH 7. Consequently, we believe that tissue decalcification achieved by this method can be used as a routine technique for microspectrophotometric studies of DNA in calcified tissues.

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TABLE I

	N		DNA amount
HEPATOCYTES	50	diploid	2.61 ± 0.20
	40	tetraploid	5.59 ± 0.34
LYMPHOCYTES	50	diploid	2.52 ± 0.17
CONECTIVE CELLS	50	diploid	2.59 ± 0.19

Cytophotometric values in different cell types subjected to conventional techniques for studying DNA by means of Feulgen's nuclear reaction.

TABLE II

	N		DNA amount
HEPATOCYTES	50	diploid	2.65 ± 0.29
	40	tetraploid	5.63 ± 0.44
LYMPHOCYTES	60	diploid	2.66 ± 0.26
CONECTIVE CELLS	40	diploid	2.74 ± 0.30
OSTEOCYTES	60	diploid	2.75 ± 0.28
EPITHELIAL CELLS	30	diploid	2.83 ± 0.32
ODONTOBLASTS	30	diploid	2.81 ± 0.29

Cytophotometric values of DNA in different cell types decalcified with EDTA tris pH 7 and subsequently stained with Feulgen's technique.

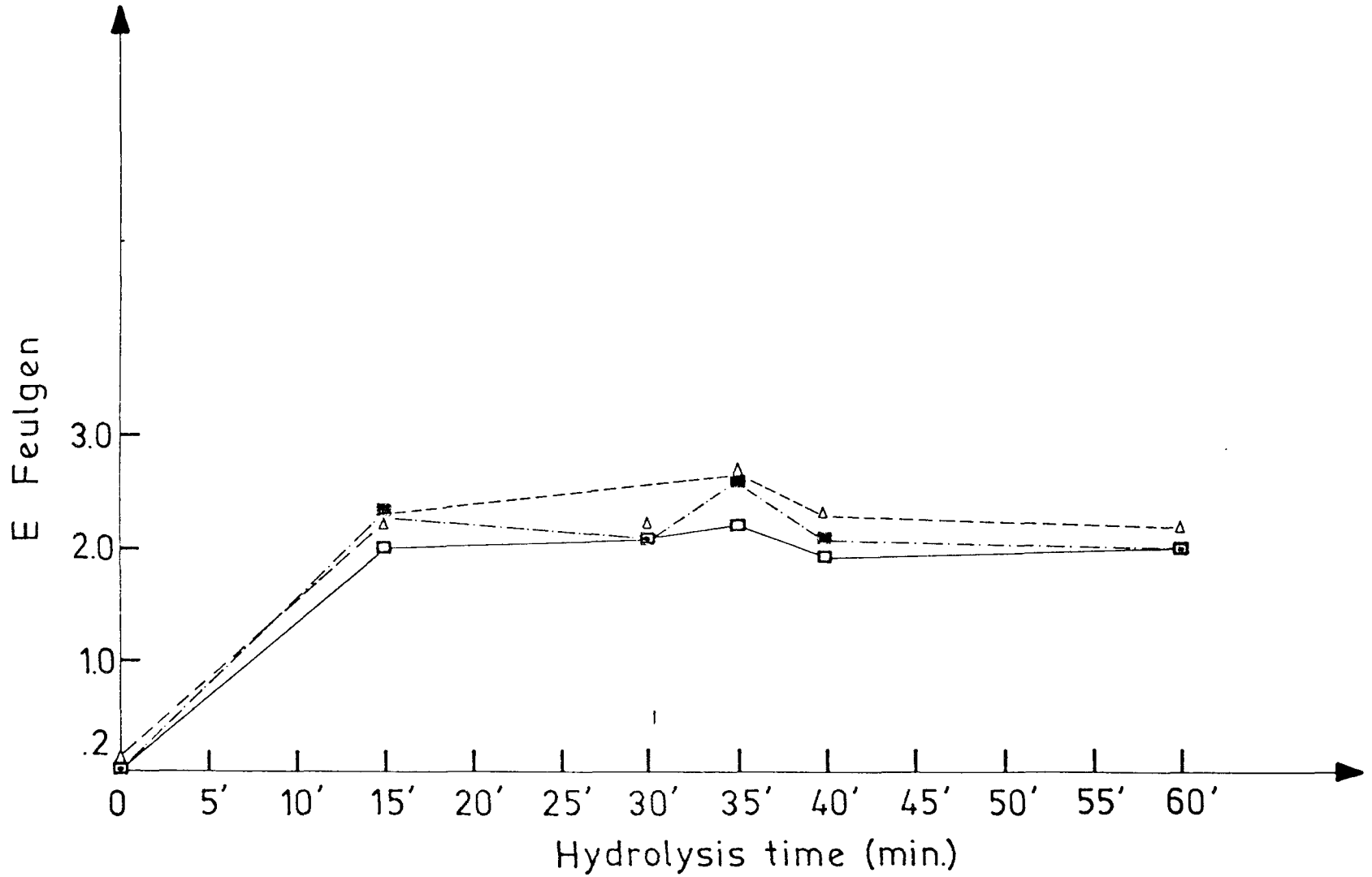

Figure 1.- Feulgen hydrolysis curves for control spleen and for bone and spleen treated with EDTA tris.

F. 17 DNA Microspectrophotometry in Human Pathology

I.B. Giménez, C.J. Conti, R.L. Cabrini, F. Schajowicz,* M.E. Solanot** and B. Lema**.

The laboratory of Pathology of the Department of Radiobiology has analyzed several pathological entities by quantitative techniques at the histological level (1-2).

This techniques have been used in pathology in order to obtain information for an early and faster diagnosis of certain neoplasic or preneoplasic lesions (3).

This program was carried out in collaboration with several Pathology Departmens using several normal and diseased tissues.

The studied material was processed in the same way: fixation in formaldehyde embedding in paraffin and staining with the Feulgen reaction. Quantitative determinations were carried out in a Zeiss Cytoscan Microspectrophotometer with authomatic scanning stage using monochromatic light of 560 nm.

Oral Lesions (Fig. 1)

The DNA of the epithelial basal cells of oral lesions (radicular cyst, keratocysts, leukoplakias and epidermoid carcinomas) was determined by Feulgen microspectrophotometry. The DNA histograms of the radicular cysts showed a definite peak in the diploid range and a slight deviation to the right. The keratocysts also showed a well defined modal peak in the diploid range, but another cell population was found exhibiting a peak in the tetraploid range.

In leukoplakias of the oral cavity two different profiles of DNA distribution were found. Where both the clinical and microscopic traits defined a non-dysplastic lesion, the histograms showed a diploid peak and also a percentage of cells in the diploid-tetraploid range. For the more active lesions a shift towards higher values was evident.

The microspectrophotometric studies of oral epidermoid carcinoma showed a broad distribution toward the right and ill-defined modal values.

Cartilage tumors (Fig. 2)

In order to establish the grade of malignancy of cartilage tumors, DNA histophotometric studies of series of 4 Chondromas and 6 Chondrosarcomas of different grades of malignancy have been performed comparing the results obtained with histopathological features.

Chondromas showed a diploid modal peak (similar to that of lymphocytes) with very litle dispersion of values. Chondrosarcomas behaved differently according to their grade of malignancy. These of grade I (low grade) also showed a modal values at the diploid level, but unlike the chondromas the histograms had a rightward asimmetry caused by an increment in proliferative cells. The modal value of the histograms of grada II chondrosarcomas differed from the diploid value and also presented an asymmetric distribution. Finally, grade III tumors exhibited two non-diploid modal peaks.

Squamous metaplasia of the cervix uteri (Fig. 3)

Seven cases of complete and incomplete squamous metaplasia were studied

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** Department of Pathology, Rivadavia Hospital, Buenos Aires.

in order to establish possible DNA-pattern alterations and to correlate them with those observed in displasias and carcinomas.

The material was obtained through biopsies and surgical specimens (hysterectomy). 100 cells were measured in each case.

DNA values of normal squamous cells showed an unimodal distribution with a peak located close to that of the lymphocytes (Fig. 1).

In metaplasic cells the distribution is quite similar to the normal one but exhibits a slight asimmetry to the right which suggests a second modal value at premitotic level.

Studies of nuclear DNA in several tumors have detected aneuploidy, poliploidy and scattering of values. Furthemore, attempts have been carried out to correlate the DNA alteration with the tumors prognosis. In our study this correlation could be seen in oral leukoplakia and in cartilage tumors. In these tumors several DNA patterns were found, from normal unimodal distribution in chondromas to aneuploidy, bimodal, distribution in grade III Chondrosarcomas. In oral leukoplakias a similar behaviour was found.

Radicular cysts and Keratocysts are two oral lesions which although presenting very similar histological features differ markedly in their evolution.

In the present study parameters were found which allowed to distinguish them from the quantitative histochemical point of view.

Squamous metaplasia of uterine cervix showed a DNA distribution resembling that of the normal tissues with well defined diploid modal values although presenting a higher activity (right asimmetry). The analysis of the data of the present study suggests the possibility of using DNA microspectrophotometry as a diagnostic tool which could be employed in those lesions with similar histologic features but different evolution and potential.

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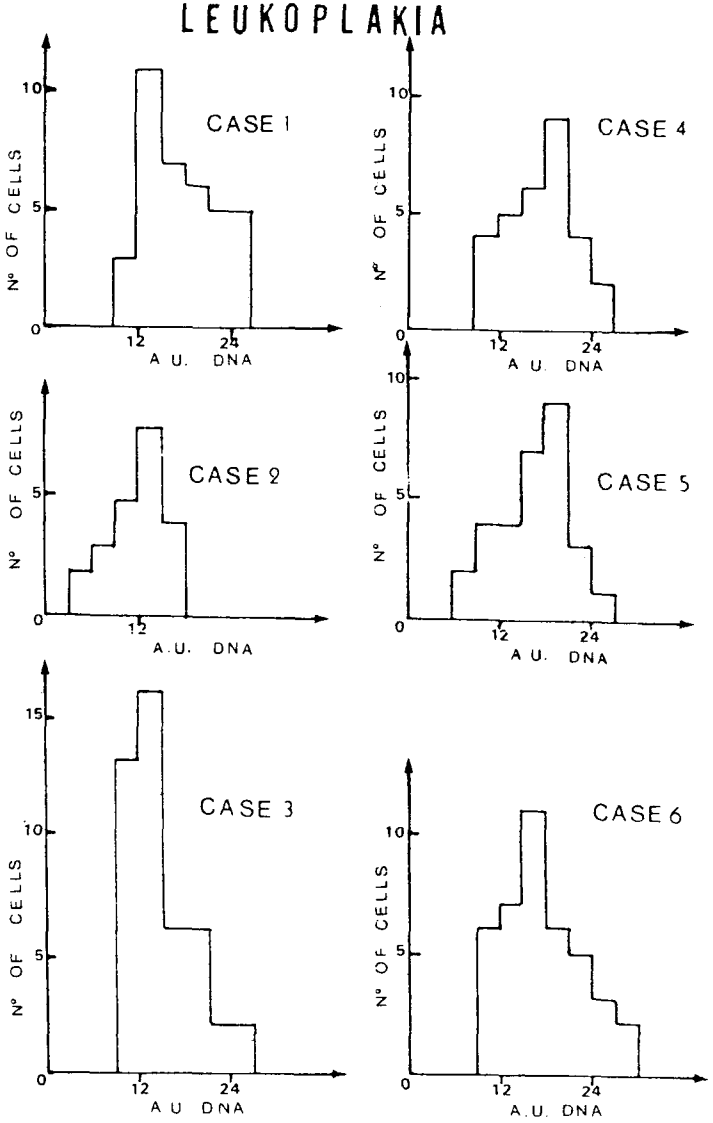
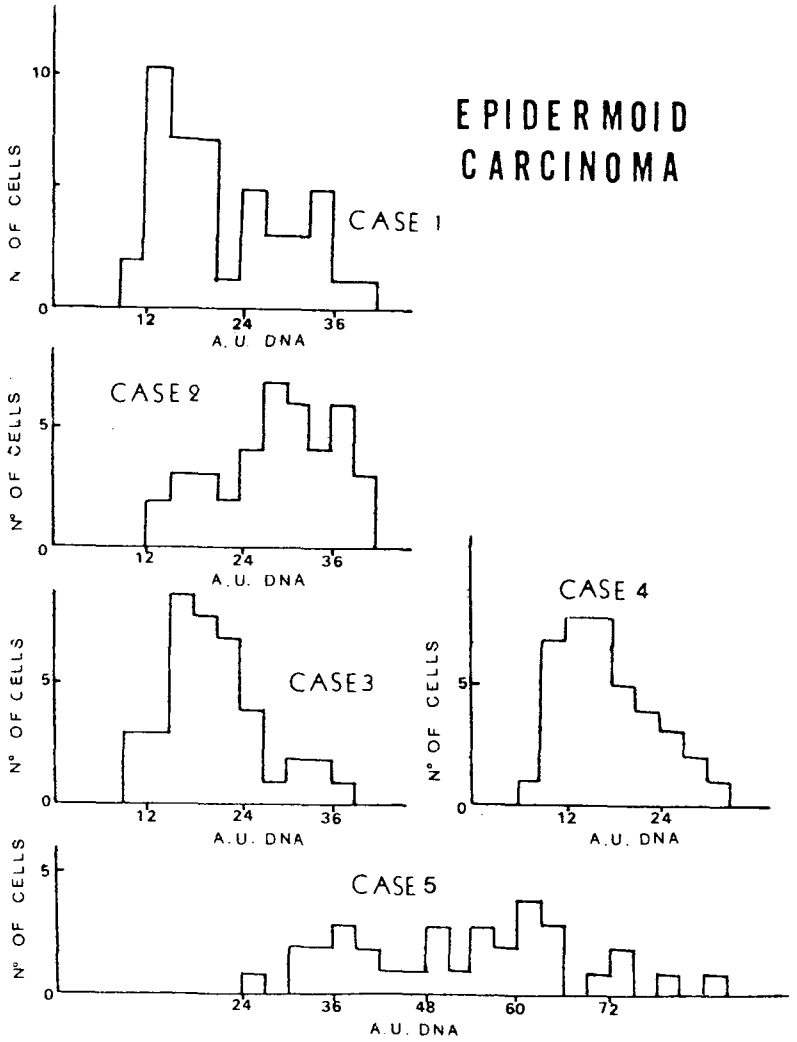
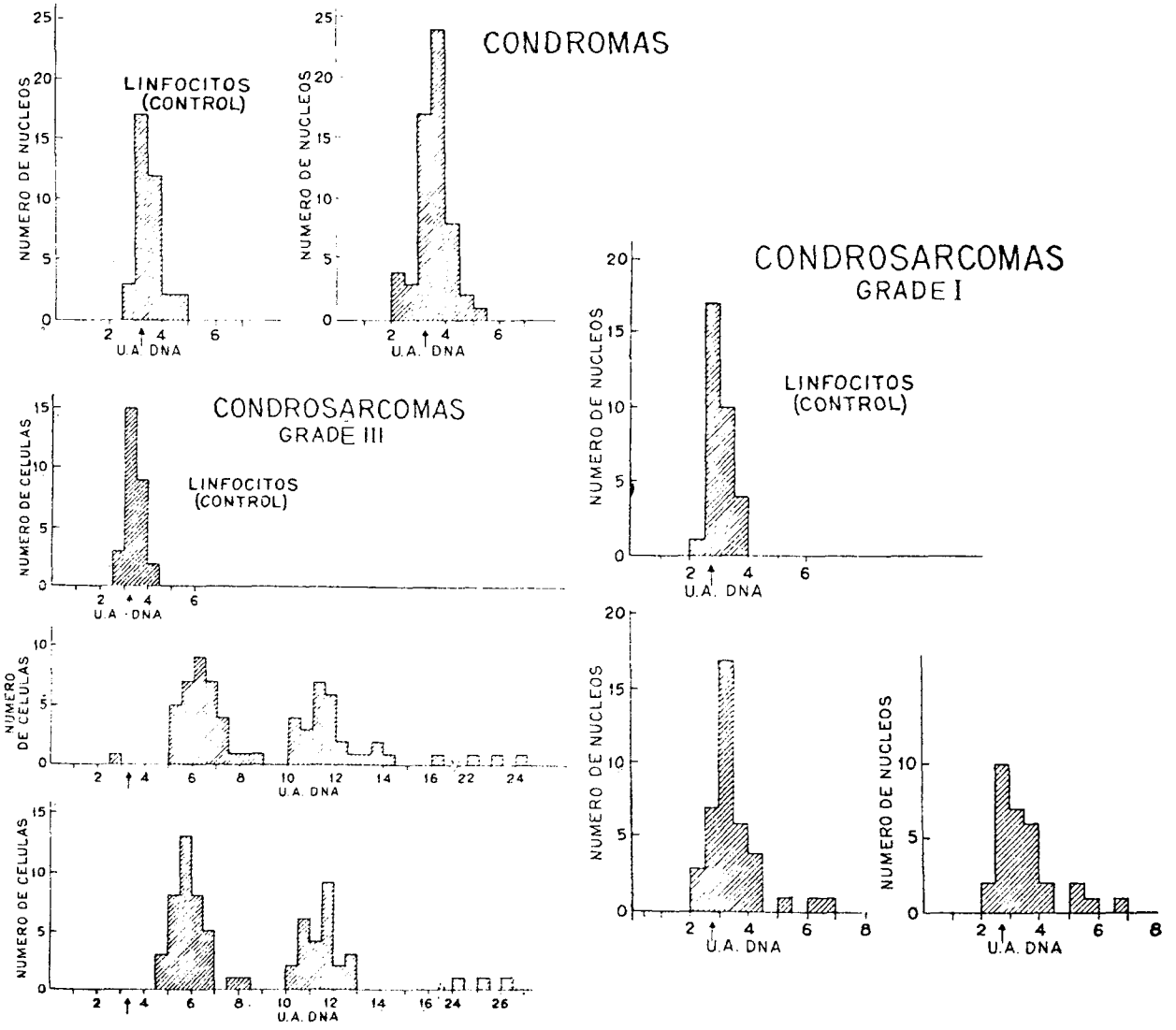
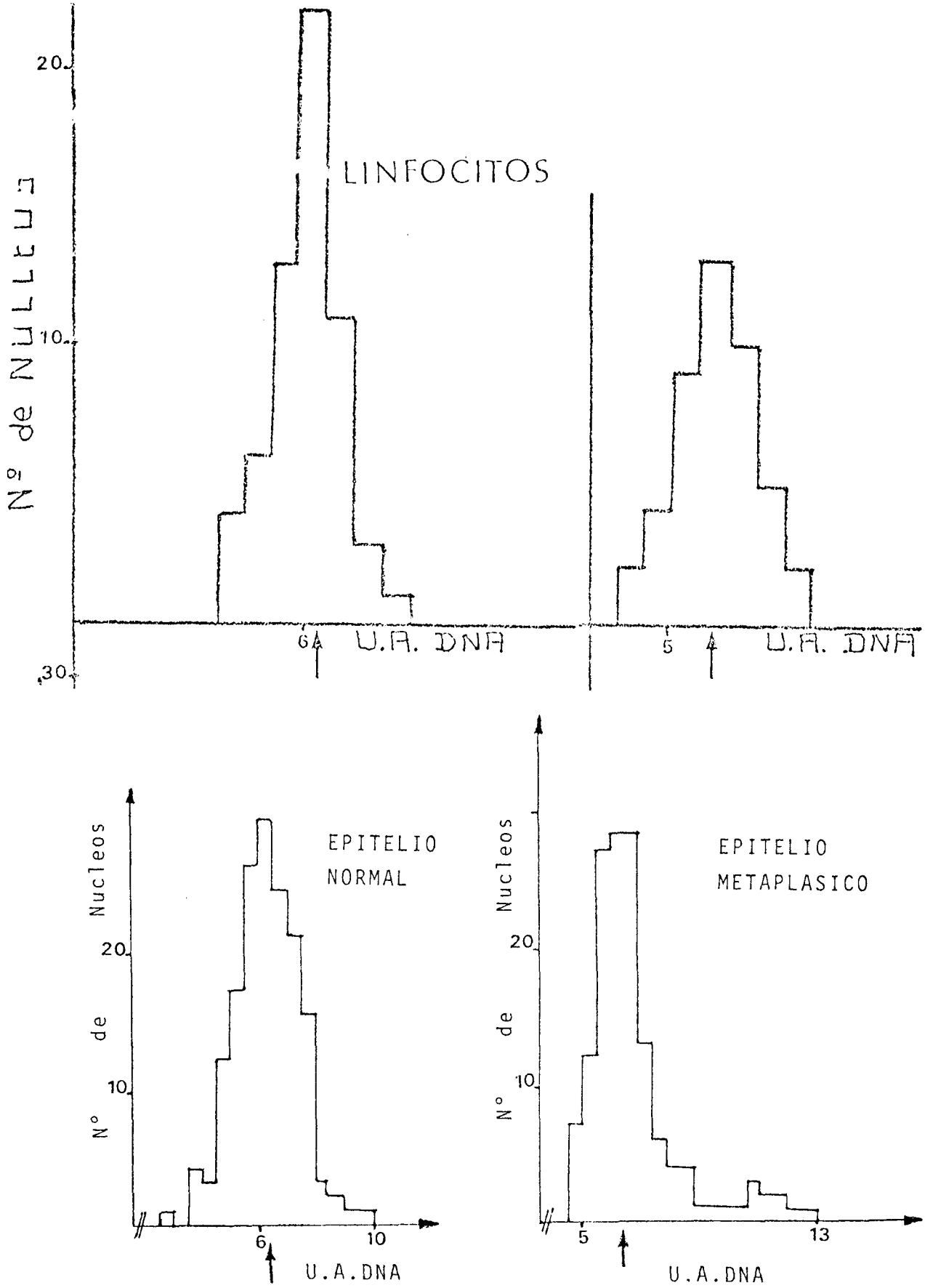
The present study was carried out with the collaboration of the Pathology Department of the Dental School of the University of Buenos Aires, The Latin-american Registry of Bone Pathology, and the Department of Pathology, Rivadavia Hospital, Buenos Aires.


LEUKOPLAKIA
CASE 1
CASE 4
CASE 2
CASE 5
CASE 3
CASE 6
N° OF CELLS
A.U. DNA


EPIDERMOID CARCINOMA
CASE 1
CASE 2
CASE 3
CASE 4
CASE 5
N° OF CELLS
A.U. DNA


LINFOCITOS
(CONTROL)
NUMERO DE NUCLEOS
U.A. DNA
CONDROMAS
CONDROSARCOMAS
GRADE III
LINFOCITOS
(CONTROL)
NUMERO DE CELULAS
U.A. DNA
CONDROSARCOMAS
GRADE I
LINFOCITOS
(CONTROL)

F. 53


LINFOCITOS
Nº de NUCLEOS
20
10
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U.A. DNA
5
U.A. DNA
EPITELIO
NORMAL
N° de Nucleos
20
10
6
10
U.A.DNA
EPITELIO
METAPLASICO
N° de Nucleos
20
10
5
13
U.A.DNA

Figura 3

F. 18 A Quantitative Histophotometric Study of the Lead Precipitation Reaction for the Demonstration of Acid Phosphatase

R.L. Cabrini, A.C.C. Frasch and M.E. Itoiz.

Microspectrophotometry in the quantitation of histochemical reactions has received a lot of attention because of its practicability, once the prior conditions of validity have been determined. Having been developed for the quantitation of the nucleic acids (Ris & Mirsky, 1949); it has now been applied to enzyme histochemistry and, by this method, oxidative and hydrolytic enzyme activity has been successfully determined (Cabrini et al., 1969).

No information is available in the literature about the application of the microspectrophotometric method to the quantitation of acid phosphatase activity demonstrated by a Gomori-type lead precipitation technique. Because of the applicability of this method to the demonstration of acid phosphatase at the ultrastructural level it has lately received preferential attention. For this reason, it seemed worth while to determine the conditions under which it can be submitted to microspectrophotometric analysis.

Two kinds of model systems were used: (a) frozen sections of Wistar rat kidney and human prostate gland; (b) polyacrylamide in to which variable quantities of total homogenate of human prostate gland were incorporated.

The rat kidneys were fixed in neutral formaldehyde solution for a period of 24 hr at 4°C, and 40 µm sections were cut in a cryostat. They were incubated for 3, 6, 9, 12, 15, 18, 21 and 24 min in Gomori's (1950) medium.

The prostate glands sections were incubated for periods of 3, 6, 9, and 12 min. The enzymatic activity of the homogenate was assayed biochemically by means of the Andersch method (1947) with a kit provided by the boehringer-Mannheim Co.

The polyacrylamide was prepared according to van Duijn et al (1967) by mixing acrylamide and bis-acrylamide with activators and accelerators to aid polymerization. The prostate homogenate, undiluted and diluted to 6, 12, 18, 24 and 30% in distilled water, was mixed with the polyacrylamide preparation before adding the accelerator. After the accelerator was added, the mixture was compressed between two glasses, which were separated by 145 µm thick coverslips. All films in each experiment were prepared on the same glass in order to avoid differences in thickness. As the results showed a good linearity, no other correction for the differences in thickness was performed.

After being polymerized, the polyacrylamide models were incubated for variable periods in Gomori's medium in the same way as the tissue sections.

Control reactions

(a) Polyacrylamide models without homogenate were incubated simultaneously with the enzyme models for different periods in order to eliminate the unspecific precipitation of lead sulphide as a possible cause of error.

(b) The phosphatase activity of the polyacrylamide models was inhibited uspecifically with 1% trichloroacetic acid for 10 min prior to incubation.

(c) 0.01 M sodium fluoride was incorporated in the incubation solution as a specific inhibitor of the acid phosphatase activity.

Microspectrophotometry

The measurements were performed in a Zeiss Cytoscan with automatic scanning stage.

Before measuring, an absorption spectrum of the lead sulphide precipitate was obtained between 470 and 660 nm on one of the polyacrylamide models. As can be seen in Fig. 1, there is no peak of absorption. Therefore, it was decided to carry out measurements subsequently at 550 nm.

The microspectrophotometric determinations of the models were performed by means of sacannings programmed with a meander pattern on approximately 150 by 150 μm areas. The total magnification was x 60 and the final values were registered as the integral of the optical densities obtained during the scanning. Zones of the renal cortex were examined at random for the experiments on kidneys, and areas of glandular acinus chosen in the prostate gland.

All experiments were repeated five times taking one piece of film per point. One area, 150 by 150 μm was scanned in each film. Each point on the curves corresponded to the integrated values automatically registered by the little spot during the scanning of the 150 by 150 μm area. The linear correlation coefficient was calculated for each experiment.

A linear relationship between microspectrophotometric measurements and incubation time was found in all the models. In Fig. 2, the values obtained for renal cortex and human prostate gland are shown. In the polyacrylamide models this linear relation was demonstrated using a fixed quantity of incorporated homogenate, incubated for different periods. This correlation is maintained up to the 60 min incubation. After this, the optical densities become too high to be registered without serious error by the cytophotometer.

The possibility that increased incubation time would intensify the polyacrylamide staining was investigated by using models incubated simultaneously with and without incorporated homogenate. In the homogenate-free models, a constant background reading was obtained for all the incubation periods (Fig. 3).

Biochemically, determinations of the extinction (acid phosphatase activity) as function of the concentration of the prostate gland homogenate in fixed volumes of distilled water indicated a linear relationship (Fig. 4a). In the same way, increasing concentrations of the homogenate incorporated in the polyacrylamide films gave proportional microspectrophometric measurements of the histochemical reaction (Fig. 4b). The range of the linear correlation coefficient, r, was 0.996-0.999; p-values were $< 0{,}001$ in all cases.

Finally, addition of sodium fluoride to the incubation medium led to no colour being produced in the polyacrylamide film and to constant cytophotometric readings irrespective of the incubation time (Fig. 5).

In these experiments, the linear relationship between optical densities and the enzyme concentrations seems enough to justify the direct cytophotometric measurement of the sulphide precipitation reaction. In our opinion, the specificity of the reaction for acid phosphatase is proven by the inhibition with sodium fluoride, and by the biochemical assays performed with a method currently used in medical practice.

Equipment able to scan extensive areas with a small spot is very useful for eliminating the distributional error, one of the most important sources of error in measuring inhomogeneous precipitates such as lead sulphide, because it is impossible to apply the double wavelenght method as the extinctions are similar over a very wide range of the spectrum.

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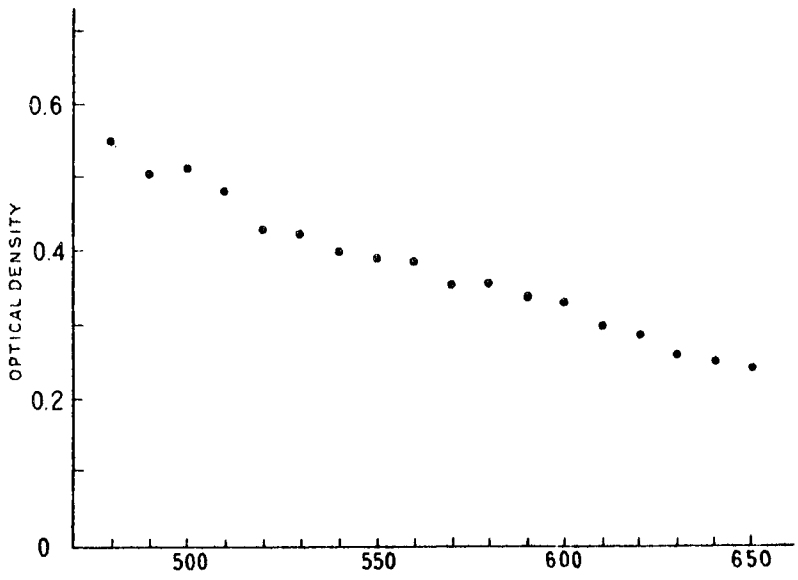

Figure 1.- Absorption spectrum obtained from a polyacrylamide model with prostate gland homogenate added in equal portions before polymerization. Reaction for acid phosphatase, 20 min incubation period.

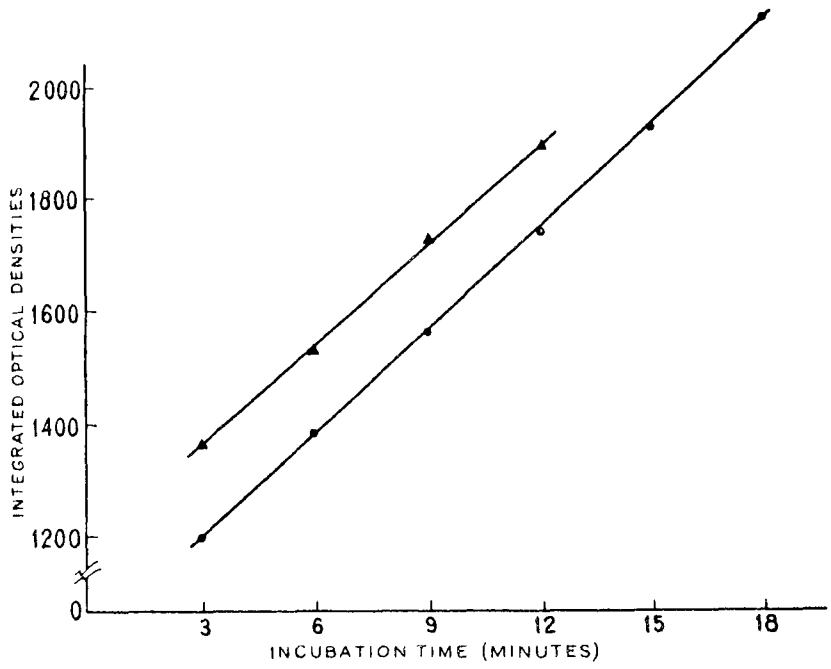

Figure 2.- Acid phosphatase in sections of prostate gland (▲) and kidney (●). Integration values of the optical densities plotted against different incubation times.

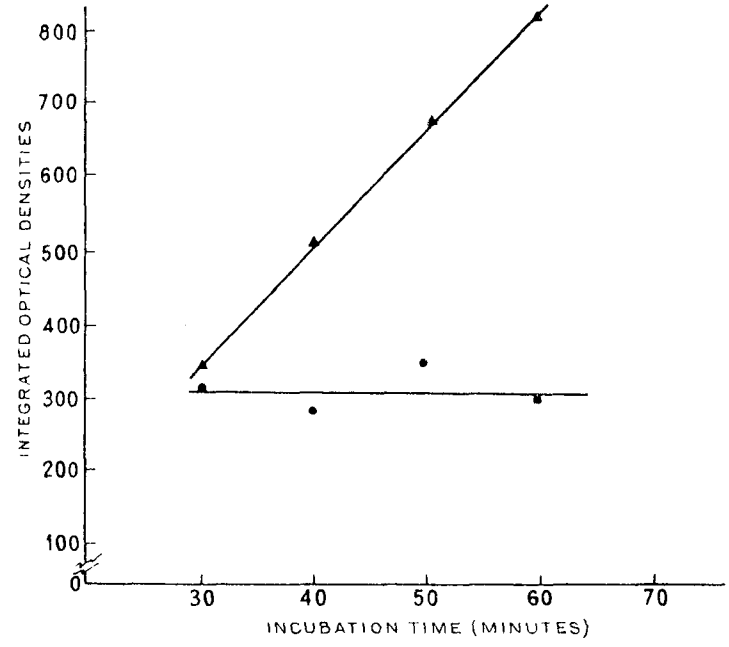

Figure 3.- Acid phosphatase reaction in polyacrylamide models incubated for 30, 40, 50 and 60 min. The microspectrophotometric measurements show a linear relationship between the optical densities and incubation times when the homogenate is incosporated (▲) and with a constant background reading in the controls containing no homogenate (●).

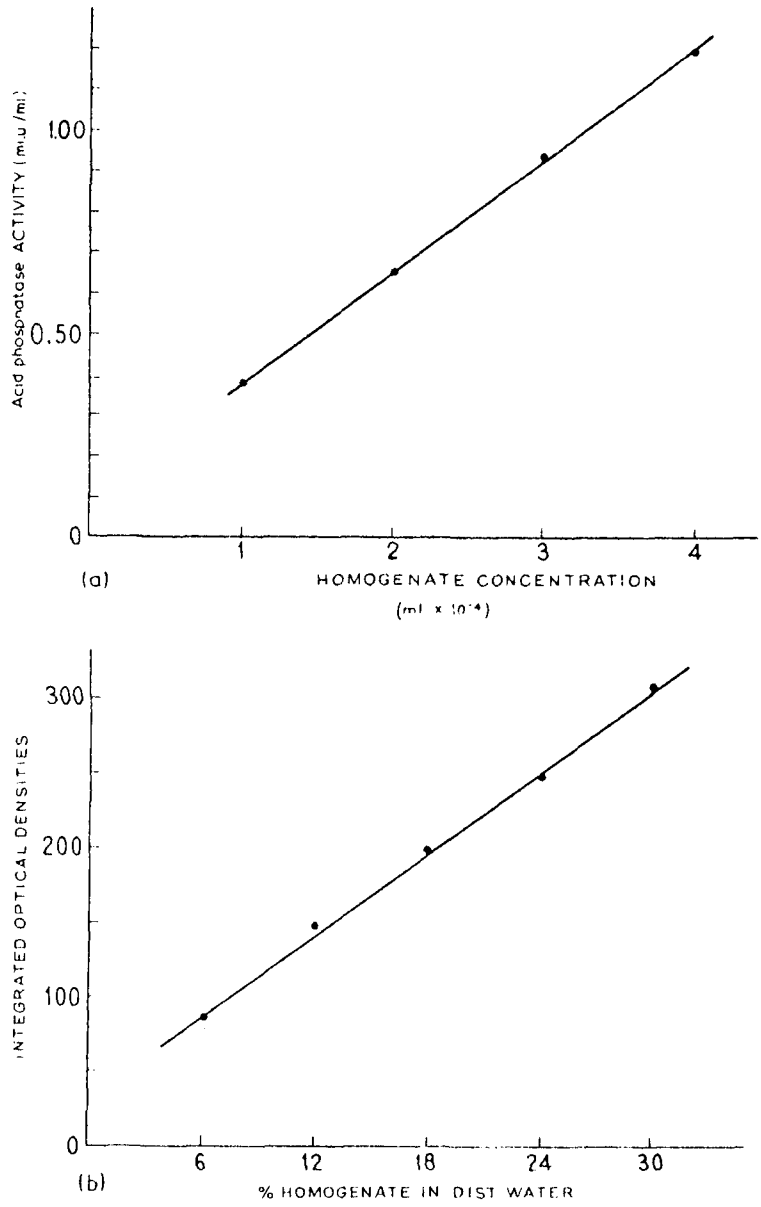

Figure 4.- (a) Biochemical assay of acid phosphatase activity with increasing concentration of homogenate in distilled water. (b) Microspectrophotometric values obtained from polyacrylamide models with variable concentrations of prostate gland homogenate.

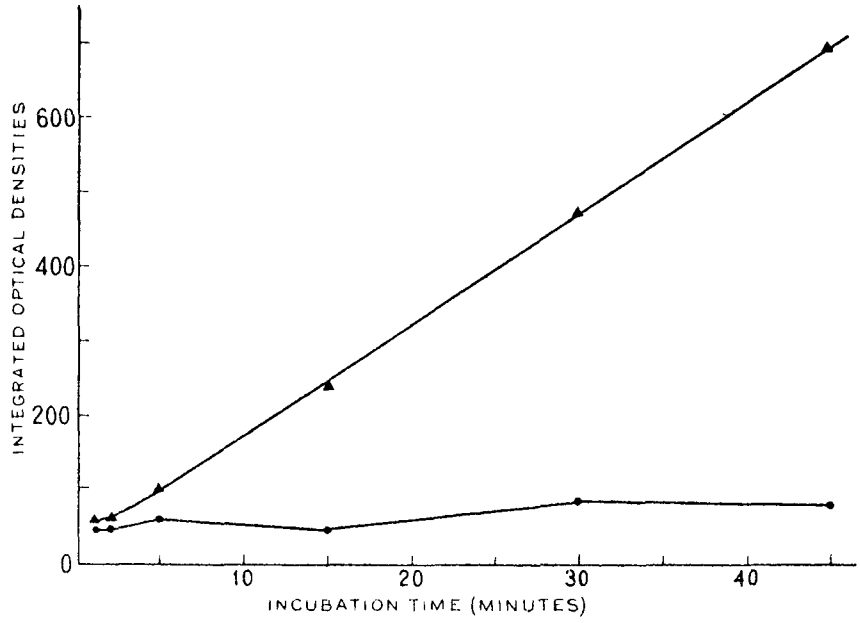

Figure 5.- Acid phosphatase reaction in polyacrylamide models with the same activity incubated for 1-45 min. The linearity of the measurements can be seen after 5 min incubation (▲). Incubation of models with sodium fluoride gave equal readings at all times (●).

F. 19 Quantitative Histochemistry of Inespecific Esterases "In Situ"

R.L. Cabrini, A.C.C. Frasch and M.E. Itoiz.

Non-haemolyzed bovine serum, kept at -20°C, was used as an enzyme source. The method of Burlina and Galzina (1) which demostrates the activities of aryl esterase was applied to determine the esterase activity of serum (3650 IU).

The models used in histochemistry consisted of the solution of different serum concentrated in a 7.5 % acrylamide preparation in phosphate buffer 0.1 M, pH 7.2. This solution was polymerized between two glass plates with a 240 ± 5 µ width spacer at 4°C. Once polymerized, the Gomori.s (2) technique for demonstrating the unspecific esterases, was carried out.

The microspectrophotometric measurements were performed in a Zeiss Cytoscan. The total magnification was x 14.3 and the wave lenght 540 nm, previously determined with an absorption spectrum (Fig. 1).

Fig. 1 shows the product (in optical density) as function of the incubation time for each one of the used serum concentrations. The reaction rates corresponding to the different serum concentrations can be noted in Fig. 2. These results indicated the validity of the microspectrophotometric quantitation of the esterase reaction.

The histochemical method, under our experimental conditions, revealed a linear response between 9 and 22 minutes. Shorts incubation periods produced non-linear patterns, probably due to the difficult penetration of substance into the polyacrylamide model.

In the histochemical method, the absorbance change was calculated per minute at 540 nm, between the 9th and 22nd minutes corresponding to the linear zone of the reaction.

1.- Burlina, A. and Galzigna, L., Clin. Chim. Acta, 39:255, 1972.

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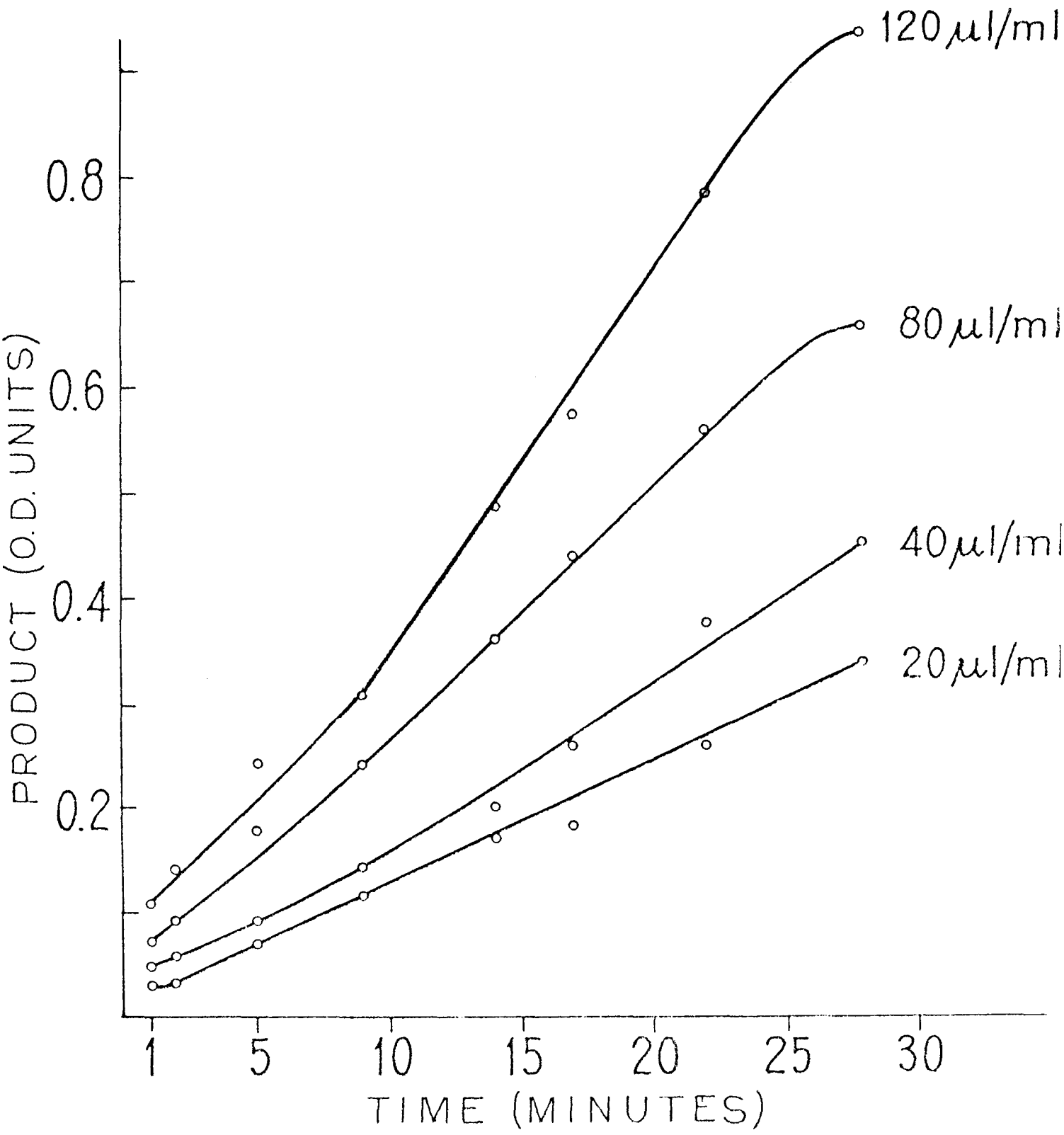

Figure 1.- Microspectrophotometric measurements (in optical density) of the yielded product as function of the incubation time. Polyacrylamide models with different serum concentrations, in which Gomori's histochemical technique was carried out to demonstrated esterases.

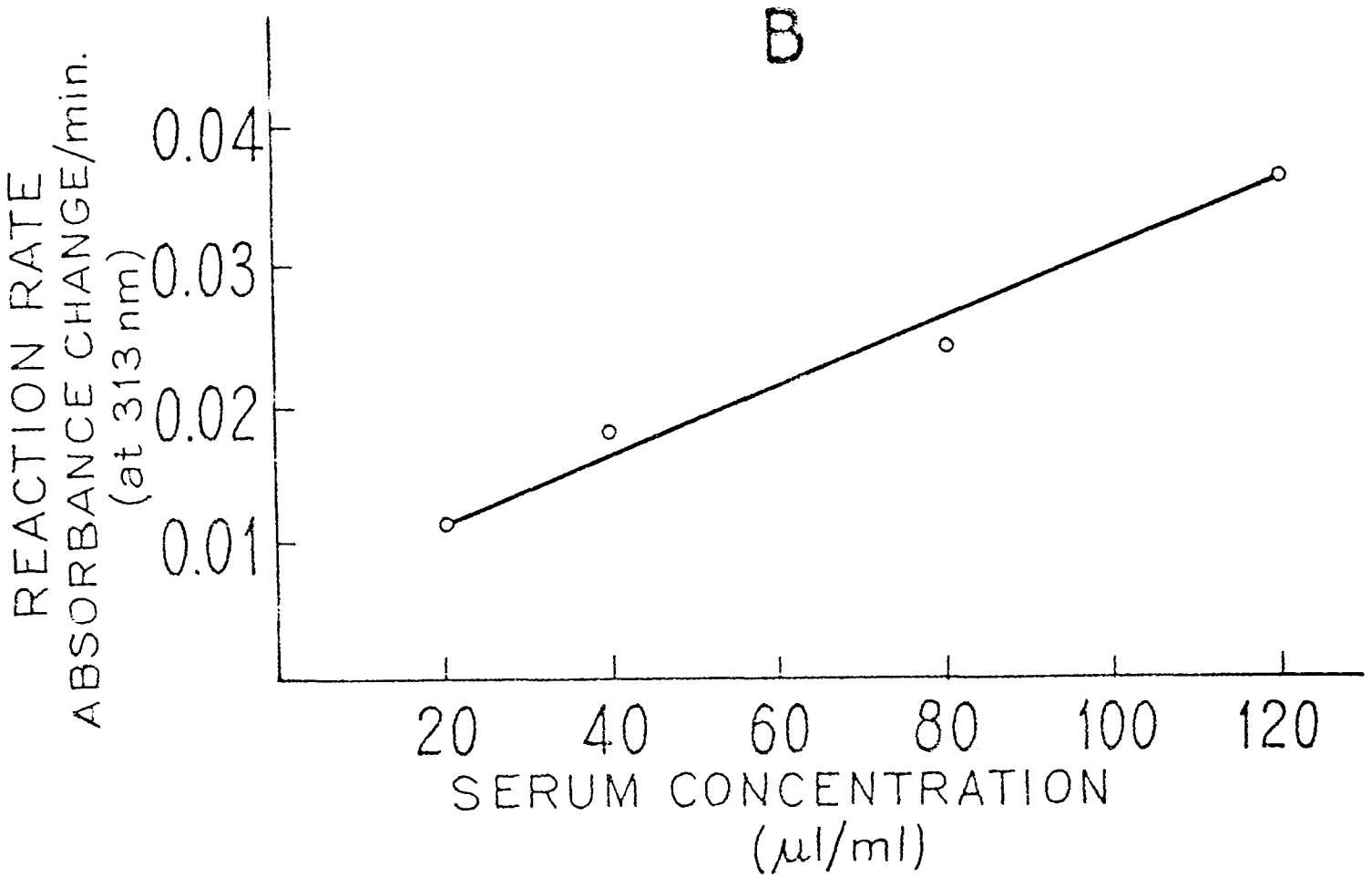

Figure 2.- Histochemical reaction rate (in absorption change at 540 nm/min) in function of the serum concentration incorporated into the polyacrylamide.

F. 20 Microphotometric Methods for Autoradiographic Quantitation

A.M. Ubios and R.L. Cabrini.

The quantitative evaluation of autoradiographic images is of great importance for an objetive interpretation. As the traditional methods of grain counting (1) are imprecise and tedious, we have adapted a microphotometer for its use.

Three diferent types of sensitive material were used: Stripping film AR10, NTB3 Emulsion and positive photographic film. The size of the silver grains of each of then was determined. For the microphotometric measurements the Zeiss Cytoscan microphotometer was used. The number of grains was counted in each measured area. The correlation between the number of grains and the microphotometric areas was established. Microautoradiographies of tissue sections stained with hematoxyline-eosin, from animals injected with different activities of tritiated thymidine (1-5-10 and 30 µC/g of body weight) were also studied.

Grain size was uniform in each material: Stripping Film 0.5 µ ± 0.1, Emulsion 0.6 µ ± 01 and photographic film 0.6 µ to 01. For measured area of 64 μ^2 the microphotometer started to discrimate up to 8 grains per field. The measurements were proportional to the number of grains for each material: Stripping Film $r = 0.951$ $p < 0.001$, Emulsion $r = 0.975$ $p < 0.001$ and photographic film $r = 0.963$ $p < 0.001$ form. Tissue section determinations shows the effectiveness of the method applied to stained microautoradiographies (Table I).

The validity of the microphotometric measurement technique is assured by the regularity of the grain size. Autoradiographic quantitation is theoretically based on the direct relationship between the amount of developed grains and the radioactivity present in the samples (2). The proportionality of our measurements in relation to the number of grains in the same field prove the validity of our method. In addition this technique is easy, quick and sensitive. The measurements obtained from autoradiographies of skin sections have demonstrated the proportionality of the microphotometric quantitation according to the initial injected activity their data is in agreement with that of a previous paper (3) in which the autoradiographic answer was compared with measurements obtained in a liquid scintillation counter (3).

The possibility of autoradiography measurement, in which the localization pattern of the labeled compound (phosphorus, fluorine, histidine) makes difficult other type of quantitation opens an exciting field. As subjective factors are eliminated, quantitation will be safer and easier.

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2.- Herz, R.H., Lab. Invest., 8:71, 1959.

3.- Ubios, A.M. and Cabrini, R.L., Int. J. Appl. Rad. Isotopes, 24:311, 1973.

TABLE I

Exposure time: 14 days

	1 µC	5 µC	10 µC	30 µC
% Transm.	86.45 ± 5.83	79.25 ± 8.02	68.20 ± 7.69	49.30 ± 12.57
N° grains	5.83 ± 2.40	12.65 ± 3.85	17.20 ± 4.12	25.20 ± 5.30

Measurements obtained from tissue sections of animals labeled with different initial activities showed that the isotope uptake is proportional to the injected activity and that the microphotometric measurements (expressed in % of transmittance) are proportional to the number of grains.

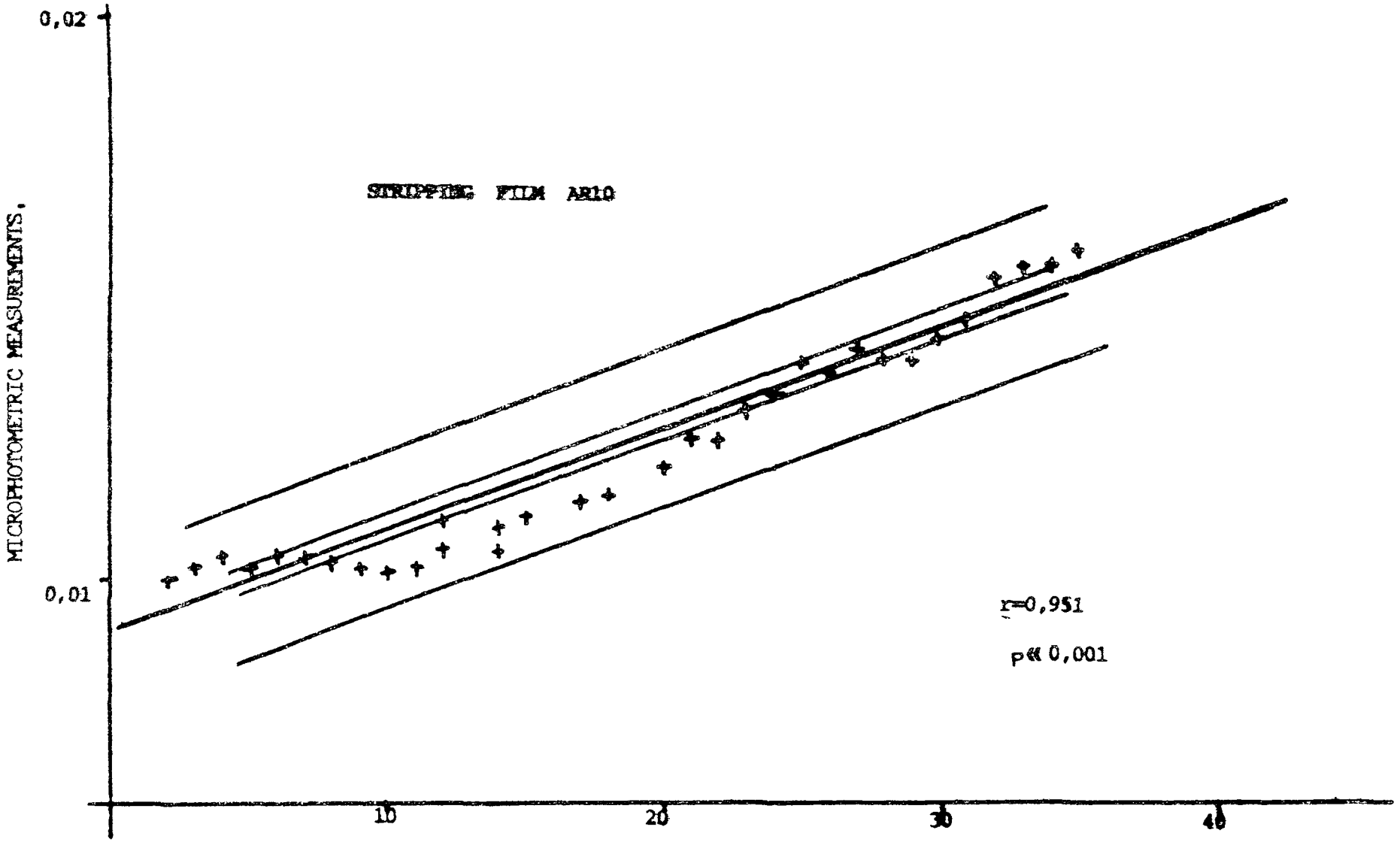

Figure 1.- Graphical representation of the correlation of microphotometric measurements (expressed as inverse of transmitance) and number of grain in a measured areas of 64 μ^2. This graph corresponds to Stripping Film AR10, NTB3 Emulsion and positive photographic film showed similar correlation values.

F. 21 Localization of ^{32}P by Autoradiographic Methods

A.M. Ubios, F.S. Silberman,* F.F. Miranda,** R.L. Cabrini and O. Degrossi**

Among the numerous therapeutically used radioactive isotopes ^{32}P as colloidal chromic phsphate (^{32}P CROP) (1), was proposed for treatment of human osteoarthrosis. The aim of this study was to improve the knowledge of the real localization of this isotope in the different zones of the articular cavity's surface.

Wistar rats of 300 g of body weight were used. The animals were injected unilateraly with 0.25 μC of ^{32}P CROP in the articular cavity. They were killed in groups after 1, 14 and 28 days. In addition dogs were also used in this experiment. Groups of them had been operated in order to provoke an experimental arthrosis of the knee 30 days after. They received 1 mC of ^{32}P CROP in the articular cavity. Another group of dogs considered as controls was also injected with 1 mC of ^{32}P CROP in the articular cavity. The animals were sacrificed in both cases 14 days after the injection.

The radioactivity of equivalent samples of synovial, meniscus and articular cartilage were measured in a 4 πcounter. Macroautoradiographies of rat knee joints and knee joints microautoradiographies of rats and dogs were prepared. Microphotometric autorediographic quantitation were made with a Zeiss Cytoscan.

After analyzing samples from different tissues it was evident that the synovial exhibited the highest activity both in control as in the experimental dogs (Table I).

Some histological changes were seen in the synovial membrane of the rats: cronic inflamation, newly formed vessels and xanthomatous cells as well as myxoid degeneration in the bone marrow. Control dogs showed no histological changes operated dogs showed typical arthrosic alterations.

The presence of the radioisotope in the articular cavity 14 and 28 days after the injection was evident in the macroautoradiographies.

Microautoradiographies from normal dogs and rats showed ^{32}P labelling on all synovial surfaces and, with lower intensity in macrophages (Fig. 1). Microautoradiographies of arthrotic dogs showed a highly irregular label of the synovial surface and in macrophages of the villi near the vessels. Isotope label was also found on altered cartilage surfaces. Microphotometric measurement showed a 3:1 ratio between synovial and cartilage isotope uptake.

Our histological findings indicate that the employed doses produced no changes in normal dogs after 14 days of the ^{32}P CROP injection in the articular cavity. In rats, probably due to the higher injected activity, some histological changes occurred.

The scintillator countings demonstrated a higher ^{32}P CROP uptake by the synovial in both control and operated dogs.

The autoradiographic methods permitted a better localization of the isotope in the joint tissues. It is very interesting that the microphotometric measurements detected a macrophage label gradient in the synovial membrane which decreased from the surface to the deeper tissues. A very interesting finding in

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** Nuclear Medicine Center, Buenos Aires.

relation to possible therapeutical consequences is the isotopic labeling on the cartilaginous surface of arthrosic dogs and the great amount of labeled cells in the villi.

Our finding could be of interest in relation to the degree of safety or danger (2) of the therapeutical uses of ^{32}P CROP and to its possible mechanisms of action in osteoarthrosis.

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2.- Dyson, E.D., Health Phys., 12:1521, 1966.

TABLE I

	Synovial	Meniscus	Cartilage Femur	Cartilage Tibia
	%	%	%	%
Non operated animals	53.11	15.97	18.47	16.33
Operated animals	68.43	3.98	2.38	24.31

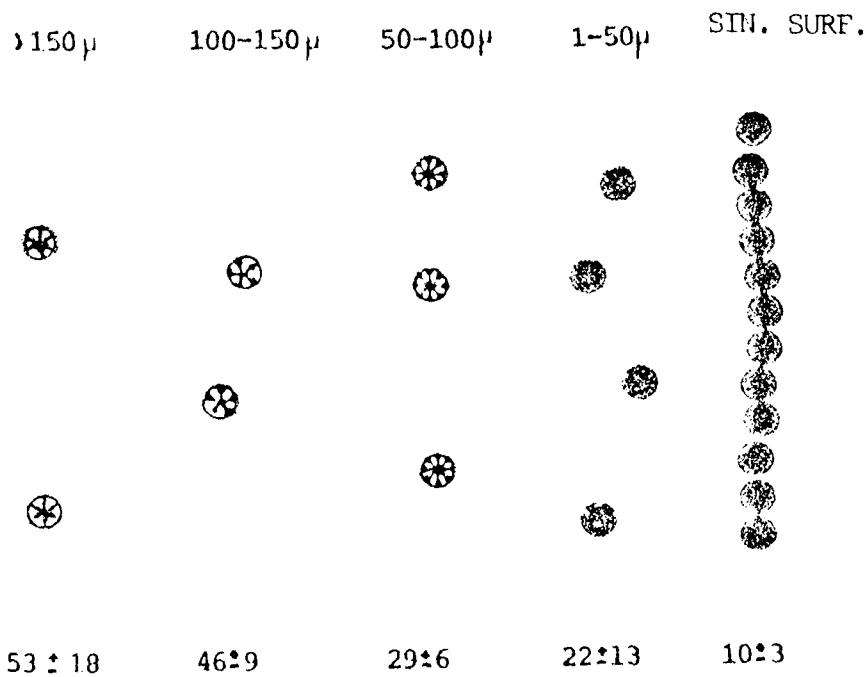
>150μ
100-150μ
50-100μ
1-50μ
SIN. SURF.
53 ± 18
46±9
29±6
22±13
10±3
% OF TRANSMITTANCE

Figure 1

F. 22 Optimization of Skin Tissue Cultures Techniques for Radiobiological Use

C.J. Conti and C. Pardini.

Several studies on the physiology and morphology of irradiated epidermis were performed in our Department in the last five years; all of them were carried out using in vivo specimens.

In order to distinguish between the effects exerted by ionizing radiation directly on the skin, and those that could be produced indirectly (through vascular injury, edema, etc.) we took into account the possibility of introducing skin culture systems in our laboratory.

Material: newborn and young rats, adult mice, adulta human and chick embryo skin were used.

Culture media: Eagle medium or TC-199 supplemented with fetal calf, calf of horse serum and antibiotics.

Culture flasks: Weaton glass flasks; or poliestyrene capsules or coverslips were employed.

Culture techniques: three major techniques must be considered, a) free explants (organ culture); b) plasma clot fixed explants and c) free cells (cell culture).

1) Fixed explants: different systems of free explants were performed, but all of them were carried out mantaining the explants in the gas-liquid interphase in order to achieve a good epidermal cell oxigenation.

2)Plasma clot explants: small pieces of skin were fixed to the botton of the culture flasks through a chicken plasma clot (plasma clots were obtained by mixing 50:50 chicken plasma and embryo extract). The clot was covered by the culture medium. The cells migrated from the margins of the explants and stuck to the bottom of the flask's surface.

3) Free cells: The epidermis separated from the dermis by trypsin incubation. After that the keratinocytes were loosened from the epidermis by mechanical treatment. The free cells were seeded in a proper flask and covered with culture medium.

1) Free explants: this kind of cultures showed very different behaviour patterns depending on the material employed (human, rat, etc.). Most of the study was carried out in rats, taking into account that these animals were used in the "in vivo" experiments. However it was not a good choice because explants of rat skin showed degenerative changes after some hours of incubation. These changes could not be delayed by using different sera, media or even by oxigenation. The addition of corticoids was also ineffective.

On the other hand this kind of explants developed a rapid outgrowth of epidermal cells engulfing the whole explants, which finally reduced the exchange of gas and nutrients. This phenomenon, called epiboly, could be seen before 24 hs.

Mouse explants, had a similar behaviour.

Human and chick embryo cultures, were more resistant under the same conditions showing few degenerative changes after several days of culture.

2) Plasma clot explants: rat and mouse explants showed an important outgrowth of cells. However, the epidermal cells appeared overgrown by the dermal fibroblasts.

3) Free cells: after 24, 48 hs "in vitro" rat epidermal cells tended to form confluent monolayers of poligonal cells. Dermal fibroblasts, when present were easily distinguished because of their characteristic morphology. We have maintained healthy keratinocyte-cultures for several weeks, and even mitotic figures could be seen in most of them.

There were no differences between cultures grown on glass or plastic surfaces. Fetal calf serum seemed to be an essential factor for cell growth. With calf and horse serum very poor results were obtained.

Degenerative changes of the mouse and rat explants makes them useless as a radiobiological tool for long and middle range experiments but they could be used just for experiments of short duration.

Clot explants seemed to be a poor source of epidermal cells because of the overgrowth of connective cells. On the other hand through of a suitable trypsin treatment a homogeneous, healthy and viable population of epidermal cells could be obtained.

Human explants (free and fixed) were widely used by several authors. However it is not ideal for radiobiological experiments as it is not homogeneous because of its surgical origin.

Embryo chick explants, in spite of having different morphologic and physiologic characterictics when compared to those of the mammalian skin, could be a good system to analyze some radiobiological phenomena and will be taken into account in our future work.

F. 23 Electron Microscopy of Polytene Chromosomes of Drosophila melanogaster

W.F. Kirschbaum.

A technique with the following characteristics was planned and developed, for the study of polytene chromosomes of Drosophila melanogaster by electron microscopy for cytogenetic analysis:

1.- Practically with one squash of embedded polytene chormosomes enough material is obtained to study a problem and enough preparated choromosomes left to repeat or pursue further studies.

2.- The possibility of selecting by light microscopy, the chromosomes or regions of chromosomes already embedded, that are to be studied by electron microscopy.

3.- To obtain sections parallel to the chromosome axis, in all its lenght, from at least one well stretched chromosome arm, or a special region that contain several chromosomes.

M. Sorsa (1) and H.D. Berendes (2) obtained sections of squashed polytene chromosomes, that only contain part of them and are only partially stretched. Therefore for the aimed purpose, those techniques could not be followed.

The technique that was developed consists of obtaining squashed chromosomes between two siliconed cover slips, using lactic acid-orcein. Afterwards the cover slips are separated with liquid N and post-fixation was performed.

This is followed by dehydration with graded methilic alcohols and staining with alcoholic solution on uranyl acetate, during the dehydration steps. After the embedding with Epon, one sticks together with the same Epon the coverslip on a plate of several milimeters thickness of Epon. Once polymerized, the coverslip is taken off with help of liquid N leaving the squashed chromosomes stuck together on the Epon plate. Finally with light microscopy one can choose the desired well stretched chromosomes, that are still easily seen by the orcein staining, cutting them out and making blocks out of them. Sectioning with the ultramicrotome is done with great care, because only the first six sections contain chromosomes, on account of their extreme thinness. Mounting the sections over grids for serial sections, images are obtained that cover a whole arm of a chromosome, maintaining continuous uninterrupted structures allowing an analysis, without confusion of regions and sequence of bands.

A technique was previously developed placing a carbon film over the cover slip by Kirschbaum W.F. and Fromme H.G. (3) to facilitate the unsticking of the coverslip from the Epon plate.

But the carbon film did not allow a good stretching of the chromosomes, although the squashes were done with siliccned slides and only the coverslip had carbon film with which the process was followed. The chromosomes with a maximum of stretching are those that show the highest resolution of bands.

Comparing the electron microscopic analysis with the original schemes obtained by light microscopy, shows that one third of the double bands could be considered as such in the arm of the 2R chromosome and only one tenth considered by 4th. chromosome. This occurs because with electron microscopy one can only consider bands as double, when they are truly separated and simultaneously maintain contact zones.

They can never be distinguished by their thickness. One can neither find

a complete coincidence of bands and for example an additional band was found at zone 60F of the 2R chromosome.

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G. 1 Section of Irradiation and Dosimetry

Activities 1975-1976

E.J. de Aisenberg and D. Prahic.

This section furnishes the necessary equipment and thecnological knowledge in order to achieve adequate irradiation and dosimetric procedures for biological purposes.

During the last two years irradiations were performed with a Philips 250/15 radiotherapy unit and a ^{90}Sr beta-ray source.

Irradiation were performed under the following conditions:

A) Whole body irradiation: 1) hamsters (290 animals)
   2) Wistar rats (100 animals)
   3) C3H,BALB,and CFW mice (730 animals)
   4) Drosophila.

B) Local irradiation: 1) tongue of Wistar rats (11 animals).
   2) skin of Wistar rats (1220 animals).
   3) Mice thymus (72 animals).
   4) Rat thyroid gland (120 animals).
   5) Skin wounds (Wistar rats) (37 animals).

C) Irradiation "in vitro": 1) Tissue cultures.
   2) Thyroid tissue slices.

D) Plant irradiation: 1) Barley seeds
   2) Citrus seeds.
   3) Corn polen.

These irradiation procedures were carried out as part of the different research programs of the Department of Radiobiology, C.N.E.A. In addition the following institutions used this service:

- School of Medicine, University of Buenos Aires.
- School of Dentistry. University of Buenos Aires.
- I.N.T.A., Castelar, Buenos Aires.
- Enviromental Protection Office. Secretary of Public Health, Buenos Aires.
- Phytothecnical Institute, La Plata, Buenos Aires.
- National Academy of Medicine, Buenos Aires.
- Leper Research Institute, Rosario, Santa Fe.

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