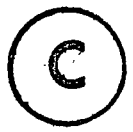
NUCLEAR RETENTION OF TOPICAL GLUCOCORTICOIDS AND ABSENCE OF CROSS-LINKING WITH DNA IN CULTURED DERMAL FIBROBLASTS

by



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ABSTRACT

The principal mechanism by which glucocorticoids exert their physiological effects is by alterations of DNA metabolism. Studies have been done to clarify the nuclear sites of human and mouse L-929 fibroblasts, with which the topical glucocorticoids, hydrocortisone (HC) and triamcinolone acetonide (TA), are likely to interact for initiating the alterations in DNA reactions, especially DNA itself and chromatin proteins. Further, data have been added on the uptake and retention of TA in the nuclei of L-929 fibroblasts. All studies have been done using actively metabolizing cells in culture over extended periods of time of exposure to the steroids (0-96 hr).

The possible direct glucocorticoid-DNA interaction by cross-linking was investigated by hydroxyapatite chromatography and thermal scanning analysis, using DNA isolated from 8-methoxypsoralen treated and UV-A irradiated mouse L-929 fibroblasts as positive controls. Cross-linking was not detectable under widely varying experimental conditions.

The fate of the glucocorticoid in nuclei was studied by measuring the amount of specifically retained TA. The specific retention of ^{3}H-TA at a concentration of 10^{-8} M in cultures of L-929 fibroblasts was evaluated in intact nuclei, chromatin and deproteinized DNA. The distribution of specifically retained TA between cytoplasm and nuclei, and its intranuclear distribution between the nucleoplasm and chromatin also were determined as a function of time. Approximately, 10% of cytosol retained TA was translocated into the nucleus. The highest level, 0.4 fmole/ug nuclear protein or 2.8 fmole/ug DNA, was reached after 6 hours. Relatively constant levels, approximately half of the highest level, were observed thereafter up to 96 hr. The majority of TA in the nucleus, 56-75%, was associated with chromatin. The level of TA retained in chromatin reached the highest level, 0.6 fmole/ug chromosomal protein or 1.1 fmole/ug DNA, after 6 hours of incubation. Relatively constant

levels,approximately 60% of the highest level, were observed thereafter up to 96 hr.

Alternative sites of subchromatin localization of the ^{3}H-TA, in terms of various classes of histone and nonhistone proteins, were studied in a preliminary investigation by the selective dissociation method of immobilized chromatin on hydroxyapatite. The TA was located in unbound chromosomal proteins (75%), histone proteins (13%) and nonhistone proteins (12%). None of the glucocorticoid was found in fractions of nucleic acids, thus confirming the unlikliness of direct glucocorticoid-DNA interactions, but indicating that the primary sites of TA-nuclear interactions reside in chromatin proteins.

The suppressive effect of triamcinolone acetonide, at concentrations of 10^{-8} and 5.0×10^{-6} M in culture media, on nuclear proteins and DNA of mouse L-929 fibroblasts was determined for various incubation intervals. The suppression was demonstrated to be a late, 24 hours or longer, consequence of the treatment of TA.

The chemical stability of the glucocorticoids used was examined in storage solutions and in media removed from test cultures by a gas-liquid chromatographic assay developed for the purpose. The assay involved double derivatization of the glucocorticoids with methoxyamine and N-trimethylsilyl imidazole, and is capable of detecting nanogram quantities of hydrocortisone, triamcinolone acetonide and desonide.

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosine 3',5'-monophosphate
C.V.	coefficient of variation
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DSC	dilute saline citrate
DSN	desonide
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FID	flame-ionization detection
GLC	gas-liquid chromatography
GC-MS	gas chromatography - mass spectrometry
HAP	hydroxyapatite chromatography
HC	hydrocortisone
HTC	hepatoma tissue culture
i.d.	internal diameter
LSC	liquid scintillation counting
MO	methoxime derivative
MOP	8-methoxypsoralen
PBS	phosphate buffered saline
PRG	progesterone
S.D.	standard deviation
S.D.S.	sodium dodecyl sulfate
S.E.	standard error
SSC	standard saline citrate
T	triamcinolone

TA	triamcinolone acetonide
TMS	trimethylsilyl derivative
UV-A	ultraviolet radiation with long wavelength, 320-400 nm

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DEDICATION

To Mom, Dad and Shiu-Leung for their confidence in me and, above all, for their love and encouragement

INTRODUCTION

Advancing knowledge in pharmaceutics indicates a progressively greater need for the application of biochemical techniques to the solution of research problems in pharmaceutics. A case in point is the problem of topical glucocorticoids.

Formulation of a topical glucocorticoid cream, for instance, involves not only a selection of the glucocorticoid, the vehicle ingredients and their concentrations, but also an evaluation of the potency of the finished preparation and its potential to produce adverse effects. At present there are only empirical potency bioassays available which are based on such gross physiologic endpoints as skin blanching, erythema, edema or thinning of the skin (Haleblian, 1976; Stoughton, 1977; Maibach and Stoughton, 1975). These bioassays relate at best indirectly to the basic mechanism of glucocorticoid action. To establish a more rational relationship between the physical chemical properties of the dosage form of a topical glucocorticoid and its clinical properties, it is necessary to know the mechanism on which the glucocorticoid action depends. However, at present, even the site within the nucleus where the glucocorticoid acts is uncertain.

Glucocorticoids act on DNA metabolism, especially transcription, to exert their physiological effects (Baxter and Rousseau, 1979). However, it is not clear whether the glucocorticoid acts by direct binding to DNA or by perturbation of some other nuclear components (Baxter and Rousseau, 1979; Higgins et al., 1979). It seemed attractive therefore to investigate whether a direct effect on DNA could be detected by studying its possible cross-linking by glucocorticoid (Duax, 1976).

The principal objective of this research has been the search for

a direct effect of glucocorticoids on DNA in intact metabolizing cells, and to gather preliminary information on alternative nuclear components which have the "specific" (high affinity) binding ability presumably necessary in initial reaction sites for the glucocorticoids.

Also, part of the research project has been the development of a quantitative gas-liquid chromatographic assay for monitoring the chemical stability of glucocorticoids during prolonged exposure to cultures of actively metabolizing cells.

LITERATURE SURVEY

I. Clinical uses and adverse effects of topical glucocorticoids

Topical glucocorticoids are by far the most commonly prescribed therapeutic agents in dermatology today. More than 50% of dermatological prescriptions are written for glucocorticoids singly or in combination with other ingredients (Stoughton, 1977). This does not mean that their use is limited to dermatologists. Rather, virtually all specialties as well as general practice, make use of these topical agents in one form or another. Now that 0.5% hydrocortisone topical preparations have been released for dispensing under pharmacists' supervision without prescription, the use and possible misuse of topical glucocorticoids are of current interest to virtually all health professions.

Topical glucocorticoids are remarkably effective for a wide variety of skin disorders. At least 56 diseases listed in Table I have been reported to be responsive to the treatment with topical glucocorticoids. Their chief effects are a profound local anti-inflammatory action, inhibition of the growth rate of epidermal and some other peripheral cells, immunosuppression and an antipruritic action. Topically applied glucocorticoids have become more and more potent with the continued development of new analogs. Refinements by substitution of fluorine, chlorine, methyl- and other groups on the steroid nucleus or by addition of various side chains have made more potent compounds either by increasing their intrinsic activity at the site of action, by promoting their speed of penetration through the skin barrier, or by delaying their metabolism to inactive derivatives. In addition, more sophisticated vehicles have increased absorption of glucocorticoids by the skin.

However, the prolonged use of topical glucocorticoids causes a number of adverse effects both systemically and locally. Systemically,

Table I Diseases with which the application of topical glucocorticoids is accepted therapy (Maddin et al., 1975)

Inflammations

Acne vulgaris
Acute radiodermatitis
Angiitis
Atopic dermatitis
Diaper dermatitis
Eczema
Lichen planus
Lichen simplex chronicus
Otitis externa
Panniculitis
Plasma cell balanitis
Polychondritis
Primary irritant contact dermatitis
Seborrheic dermatitis
Vasculitis

Immunologic Disorders

Allergic contact dermatitis
Allergic vasculitis
Berlocque dermatitis
Lupus erythematosus
Pemphigus, pemphigoid
Pyoderma gangrenosum
Urticaria
Vitiligo
Wegener's granulomatosis

Disorders of Cellular Function

Follicular keratoses
Keloid
Lichen myxedematosus
Mastocytosis
Myxoid cyst

Infections

Herpes simplex, recurrent
Impetigo
Leprosy
Rickettsial diseases
Zoster

Malignancies

Lymphoma
Mycosis fungoides

Diseases of Cellular Proliferation

Exfoliative dermatitis
Hypertrophic scar and keloid
Histiocytosis X
Keratoacanthoma
Psoriasis

Idiopathic Disorders

Alopecia areata
Aphthous stomatitis
Polyarteritis
Sarcoidosis
Scleroderma

Diseases of Neutrophil Accumulation

Subcorneal pustular Sweet's syndrome

Development defects

Epidermolysis bullosum
Ichthyosis

Metabolic Disorders

Gaucher's disease
Necrobiosis lipoidica
Myxedema

Functional Disorders

Dyshidrosis
Livedo reticularis
Pruritus

suppression of adrenal cortical function occurs from absorption through the skin to affect internal organs, especially when the steroid is used under occlusion and in children. Most currently available topical corticosteroids easily cause adrenal suppression in the majority of patients. Some, such as clobetasol propionate cause profound suppression, even without occlusion. With acute usage, the effect is transient and reversible but Carruthers et al. (1975) present evidence related to insulin stress that indicates decreased reserve occurs in patients on prolonged therapy, even though depressed cortisol levels return to normal values.

There is a good deal of patient variation, unmeasurable by any currently known properties of the corticosteroids. Some individuals demonstrate effects considerably at variance with other subjects. Also, variance occurs between compounds - some steroids show potency by granuloma inhibition and other anti-inflammation tests, but are impotent by topical application, for reasons inapparent to us at present (Stoughton, 1975). Penetration studies, as Stoughton (1975) points out, are similar between betamethasone alcohol and betamethasone-17-valerate, but therapeutic effect is radically different.

The cutaneous adverse effects are common and constitute a more vexing problem than systemic effects. Untoward cutaneous side effects include striae, erythema, telangiectasia, purpura, rosacea-like dermatitis, rebound dermatitis, epidermal atrophy and dermal atrophy. Skin (epidermal and dermal) atrophy is perhaps the most undesirable side effect of glucocorticoid treatment. The major effect microscopically occurs in the reticular dermis with degeneration of elastic fibers, collagen fibril bundle disorganization, aggregation of microfibrils and decreased numbers of fibroblasts (Jablonska et al., 1979). In severe

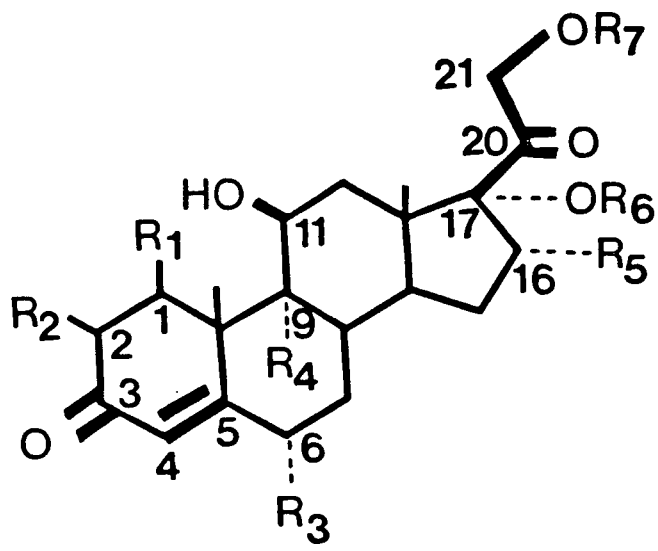
atrophy, tissue bulk will improve only about 50% after cessation of therapy (Voorhees, 1977b).

Other side effects from topical use are superinfection, addiction (Kligman and Frosh, 1979), and allergic contact dermatitis (Coskey, 1978) to the steroid compounds. Each of these poses its own problems for solution, and must be considered clinically.

Physicians must still use glucocorticoids on a trial-and-error basis (Epstein, 1979). Therefore, it would be highly desirable to establish an objective criterion for selecting glucocorticoids on a rationally determined, theoretically valid benefit/risk ratio basis. Only one assay is available for the evaluation of topical glucocorticoid potency in man, namely the vasoconstriction assay, originally developed by McKenzie and Stoughton(1962). This assay predicts reasonably well the relative clinical potencies of the glucocorticoids, but the relationship of its endpoint - skin blanching - to clinical potencies cannot be explained biochemically or physiologically. It has been demonstrated however that vasoconstriction can be correlated with concentration of steroid, and that a critical level must be present to produce it (Wallace et al., 1979). There are many vasoconstrictive drugs which have no anti-inflammatory action. The comparative assay of glucocorticoid inhibitory effect on mouse L-929 fibroblast proliferation has been used similarly to rank the glucocorticoid relative potency (Berliner, 1967a), but there are limitations in extrapolating the data to human study. Some compounds have more or less clinical anti-inflammatory activity than predicted by these assays, e.g. betamethasone valerate.

II. Structure-activity-relationship of glucocorticoids

Glucocorticoids have a 21-carbon skeleton as shown in Figure 1.



Group	Modifications
R_1 and R_2	Double bond
R_3 or R_4	F or Cl or CH_3
R_5	OH or CH_3
R_6 or R_7	Esterification
R_5 and R_6	Acetonide formation

Figure 1 : Some structural modifications of synthetic glucocorticoids

▬ : Indicates functional groups essential for glucocorticoid activity

Of major importance in steroid chemistry is the crucial relationship between structure and functional activity. Certain features are absolutely essential for the preservation of biological action of all glucocorticoid hormones. The essential functional groups are Δ^4-3-ketone, 11β-OH and 17β-ketol side chain (Foye, 1974). A number of substitutions at other sites have produced synthetic analogues with enhanced glucocorticoid activities. These modifications iclude $C_1 = C_2$ double bond, fluorination, chlorination or methylation at C_6 or C_9, hydroxylation or methylation at C_{16}, esterification of hydroxyl group at C_{17} or C_{21}, and acetonide formation with hydroxyl groups of C_{16} and C_{17} (Stewart et al., 1973).

The relative potencies of these continuously developed synthetic glucocorticoids have been reported primarily based on vasoconstriction bioassay in man and, to a lesser degree, on clinical studies. However, there are too many significant gaps in experimental and clinical data to support any firmer ranking. Moreover, the rankings reviewed in different articles demonstrate considerable discrepancy (Table II). For example, those in the cases of desonide, triamcinolone acetonide, betamethasone-17-valerate and fluocinolone acetonide. This, again, stresses the importance of searching for more reliable, objective parameters from biochemical events which constitute the receptor model of glucocorticoid action for establishing a valid and reproducible ranking of all glucocorticoids useful in topical therapy.

Ponec et al. (1980) report their attempts to relate clinical glucocorticoid efficacy with effects on human fibroblast growth inhibition in culture. They show significant differences in potency in some compounds, that are confirmed clinically, but paradoxical responses in

Table II Relative clinical potencies of topical glucocorticoids

Glucocorticoid	Relative Ranking		
	Stewart et al., 1978 (a)	Maibach and Stoughton, 1975 (b)	Haynes and Larner, 1975 (c)
Hydrocortisone	IV	VI	1
Prednisolone	IV	VI	4
Fludrocortisone	III	-	10
Methylprednisolone	III	-	5
* Triamcinolone acetonide	III (0.1%) I (0.5%)	V (cream, foam, lotion) I (ointment)	-
Beclomethasone dipropionate	II	-	-
* Betamethasone-17-valerate	II	V (cream) I (ointment)	-
Betamethasone-17-benzoate	-	I	-
* Desonide	II	V	-
Betamethasone	-	-	25
Dexamethasone	-	-	25
Betamethasone dipropionate	I	-	-
* Fluocinolone acetonide	I	V (solution) I (cream, ointment)	-
Fluocinolone acetonide acetate	-	I	-

(a) I:strongest IV:weakest (b) I: strongest VI: weakest
(c) relative potencies in numerical values (Hydrocortisone: weakest)
* demonstrates discrepancy

other clinically active agents. They caution against extrapolating these culture results to the clinical situation.

III. Biochemical events accompanying the clinical and adverse effects in the skin

Glucocorticoids have the capacity to prevent or suppress the development of the local heat, redness, swelling and tenderness by which inflammation is recognized at the gross level of observation (Haynes and Larner, 1975). At the cellular or metabolic levels, glucocorticoids have suppressive effects on the entire inflammatory process including capillary permeability, antibody formation and the repair process (Spain, 1975). This is in contrast to non-steroidal anti-inflammatory agents, such as aspirin and salicylates, phenylbutazone, indomethacin and mefenamic acid. The non-steroidal anti-inflammatory agents inhibit the exudative rather than the proliferative phases of inflammation (Foye, 1974), their actions being limited to the inhibition of prostaglandin synthesis. Therefore, they suppress histamine- or bradykinin-induced inflammation but not that dependent on neutrophils , the latter not being influenced by prostaglandins (Wintroub, 1980).

In inflamed skin, glucocorticoids affect both the epidermis and the dermis. In the inflamed epidermis, the stratum corneum is thickened and loosely organized. Cell maturation or differentiation is incomplete, and cell proliferation is increased. In the dermis, blood vessels are markedly dilated during inflammation, and inflammatory polymorphonuclear leucocytes and mononuclear cells are found. When a glucocorticoid preparation is applied topically, the glucocorticoid affects cells in each layer of the skin as it penetrates. As a result, the rate of cell maturation is normalized, and complete cellular differentiation occurs as they

migrate to the stratum corneum. Dilated blood vessels are returned to their normal diameter. Fibroblast proliferation is suppressed and the amount of dermal collagen is reduced. Inflammatory cells then disappear. The stratum corneum becomes more compact and better organized (Voorhees, 1977b).

The cutaneous adverse effects induced by prolonged topical application of glucocorticoids are thought to be mediated, in large part, by the direct action of the steroid on the fibroblasts. One of the explanations proposed for these connective tissue manifestations, including thinning of the skin, striae and poor wound healing, is a reduction in the collagen content of the involved tissue (David et al., 1970; Sim et al., 1976; Jablonska et al., 1979). The loss of collagen content may be due to inhibition of or alteration in collagen synthesis, or the excessive degradation of previously deposited collagen as shown by the results of glucocorticoid treatment of keloid in man (Griffith, 1966; Ketchum et al., 1966; Wilson, 1965). Elastic tissue and glycosaminoglycans (Jablonska et al., 1979) are also altered in this process. Studies in animals, on the other hand, have indicated an inhibition of collagen synthesis, rather than accelerated degradation, to be responsible for these effects (Smith, 1967; Kivirikko et al., 1965).

The inhibition of collagen synthesis has been proposed due to either (a) a generalized antianabolic effect of glucocorticoids on fibroblasts (Thompson and Lippman, 1974), particularly apparent for collagen because the principal synthetic activity of the cell is devoted to this protein which normally accumulates extracellularly, or (b) an effect on the enzyme, prolyl hydroxylase, which acts as a key enzyme in the biosynthetic sequence and is said to be specifically inhibited by glucocorticoids (Cutroneo and Counts, 1975; Cutroneo et al., 1971). Ponec et al.

take issue with the latter. They did demonstrate reduced collagen formation (Ponec et al., 1979).

IV. Mechanism of glucocorticoid actions - an overview

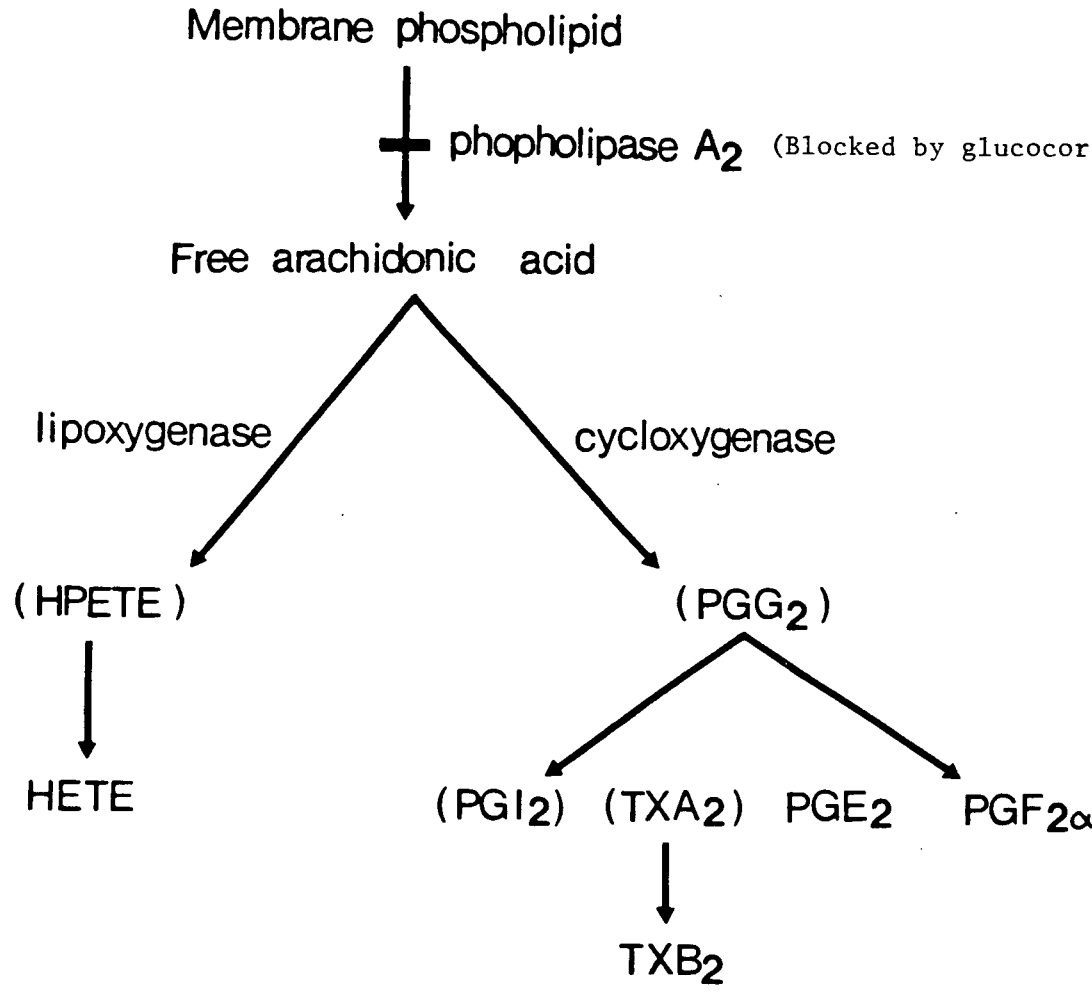
The mechanisms of anti-inflammatory action and cutaneous adverse effects are still not entirely understood, although many attempts have been made. A number of theories have been proposed to explain glucocorticoids actions since 1949 when the first glucocorticoid (cortisone) was tried for the treatment of rheumatoid arthritis (Hensch et al., 1949) and 1952 when glucocorticoid was first topically applied (Sulzberger and Witten, 1952). These mechanisms have included (1) the theory of lysosomal membrane stabilization, (2) models involving reduction of arachidonic acid and inhibition of prostaglandin synthesis, (3) the hypothesis of parallel action of cAMP and glucocorticoids, and (4) the receptor model theory.

The model of lysosomal membrane stabilization suggested that a significant portion of the therapeutic action of glucocorticoids results from a direct effect of glucocorticoids on membranes. According to this theory, glucocorticoids are incorporated into biomembranes stabilizing them by altering the movement or shape of membrane phospholipids. This stabilization has been considered responsible for the ability of glucocorticoids to inhibit the release of lysosomal enzymes and to modify the activity of other membrane-associated enzymes (Epstein, 1977; Weissman and Goldstein, 1976). Vertebrate cells that maintain cell-cell relationships, and influence their environment may do so by releasing, in soluble or particulate form, selected cell surface molecules. This process, known as shedding, is to be distinguished from secretion. Shedding is an important aspect of normal protein turnover in the cell membrane, and

proteins, lipoproteins, glycoproteins, glycolipids, glycosaminoglycans or cell surface receptors may be shed, either alone or in combinations. This process occurs in growing cells and in cells activated by various stimulants. Shedding may be affected in malignant cells by corticosteroids, and it is highly likely that these compounds affect shedding of normal cells (Black, 1980; Schimke, 1975). This affects both cell behaviour and its environment.

The second hypothesis was postulated based on the observation that topical glucocorticoids in psoriasis apparently blocked the action of the enzyme phospholipase A_2, which releases free arachidonic acid from cell membranes (Hammarstrom et al., 1977), and thereby inhibited the formation of prostaglandins, as well as the prostaglandin-related substances, the thromboxanes and 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). The sequence of events involved in this "arachidonic acid-prostaglandin transformation cascade" is shown in Figure 2. The substances synthesized in this cascade are among the most powerful bioactive compounds known and are thought to mediate inflammation including polymorphonuclear leucocyte chemotaxis. The concentration of free arachidonic acid itself and HETE were markedly increased in lesions of psoriasis (Hammarstrom et al., 1975). It was assumed that the reduction of the tissue content of these substances could be a significant aspect of the action of topical glucocorticoids in psoriasis.

The third hypothesis, now generally disregarded, states that, as the second messenger of many hormones, cAMP also mediated glucocorticoid action. It is still not settled what role cAMP plays in glucocorticoid control of cell metabolism, but it seems unlikely that cAMP mediation is necessary for glucocorticoids to act (Voorhees et al., 1975; Thompson and Lippman, 1974). Instead, it now appears that metabolic signals induced



* the compounds within brackets (PGG_2, TXA_2, PGI_2, HPETE) are unstable intermediates which have not yet been directly demonstrated in epidermis.

HPETE	12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid
HETE	12L-hydroxy-5,8,10,14-eicosatetraenoic acid
PGG_2	prostaglandin G_2, an endoperoxide intermediate
PGI_2	an unstable derivative of arachidonic acid
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

Figure 2 : Arachidonic acid - prostaglandin transformation cascade (Hammarstrom et al., 1977)

by either cAMP or glucocorticoids, affect the cell metabolism in the same directions, but by different mechanisms of action (Thompson and Lippman, 1974). As an example, glucocorticoids seem to enhance the proliferation of thymocytes induced by cAMP (Munck and Leung, 1977), and intuitively, the pharmacologic inhibition of inflammatory skin disease might be enhanced by cAMP (Voorhees, 1977a). Therefore, the therapeutic range of steroids might have been extended by cAMP.

These models of the mechanism of glucocorticoid action all have the weakness that they explain some glucocorticoid actions but not others, for instance, the antipruritic and antianabolic actions are unsatisfactorily accounted for.

The only theory that has the potential ability to explain the broader spectrum of glucocorticoid activity is the receptor model theory.

V. Receptor models of the mechanism of glucocorticoid action

Glucocorticoids differ from other hormonal steroids by affecting almost all tissues and cell types. The nature of the physiological response varies according to the cell or system being examined. The glucocorticoid mediates a large number of physiological and biochemical effects, as reviewed by Leung and Munck (1975).

The work on the mechanism of glucocorticoid actions has not advanced as far as that on sex hormones or progesterone. Enough observations testify however that the general model of steroid action, derived from studies on progesterone as depicted in Figure 3, also applies to glucocorticoids.

The "receptor" theory has been reviewed in detail in Glucocorticoid Hormone Action (Baxter and Rousseau, 1979) which contains the most recent, comprehensive reviews in chapters by Rousseau and Baxter (Chap.

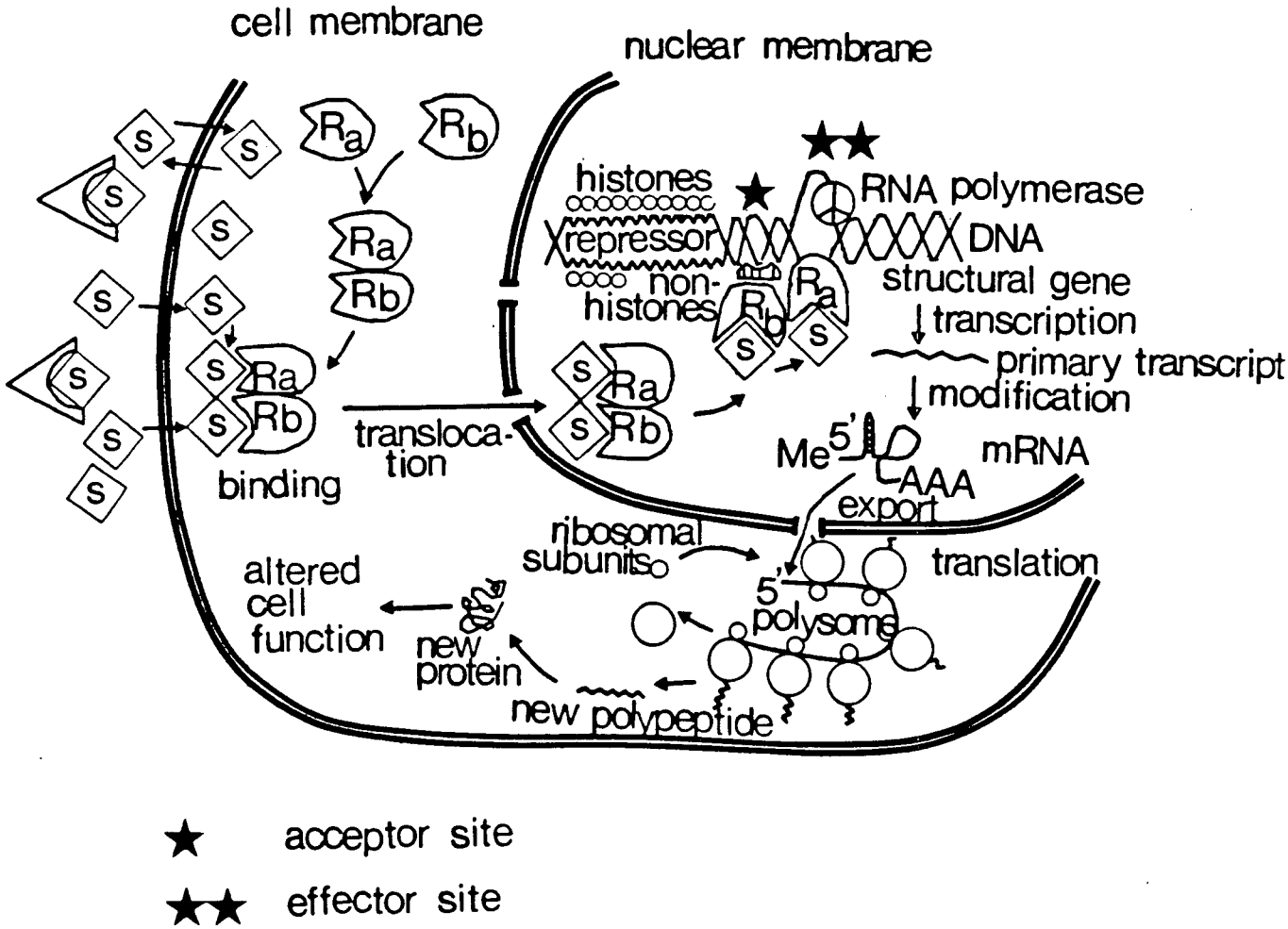


Figure 3 : The molecular mechanism of steroid hormone action (receptor model)

(O'Malley and Buller, 1977)

3), Higgins et al. (Chap. 8), Johnson et al. (Chap. 17) and Aronow (Chap. 18). Pertinent also is information in the older volumes of Receptors and Mechanisms of Action of Steroid Hormones (Pasqualini, 1976 and 1977), especially chapters 1 and 8 by Munck and Leung, and in Receptors and Hormone Action (O'Malley and Birnbaumer, 1977 and 1978) with chapters by Clark and Peck (Chap. 11) in Volume I and those by Stormshak et al. (Chap. 3), Feigelson et al. (Chap. 9), Baxter and Ivarie (Chap 10), Yamamoto and Ringold (Chap. 11) in Volume II, as well as the chapter by O'Malley and associates in Volume II (Chap. 8). Critique of the theory has been written in the Annual Review of Physiology by Gorski and Gannon (1976). Pratt (1978) has summarized the mechanism of glucocorticoid effects in fibroblasts.

The receptor model of the mechanism of the action of glucocorticoids can be summarized as follows:

(A) Non-specific binding before uptake by target cells

Naturally occurring glucocorticoids () circulate throughout the body predominantly complexed with plasma proteins, mainly with corticosteroid-binding globulin, CBG or transcortin, (approximately 77%), and slightly with albumin (about 15%). Over the normal physiologic range of 5 to 25 µg/mL for total hydrocortisone concentration in the plasma, the amount of the steroid bound to CBG increases in a nearly proportional manner to the total hydrocortisone level. The unbound hydrocortisone remains at about 8% of the total level (Tait and Burstein, 1964). Human CBG binds synthetic glucocorticoids only slightly except for prednisolone (Ballard, 1979). The distribution of glucocorticoids between unbound and protein-bound forms is dependent on both the type of glucocorticoids and the levels of plasma proteins, especially of CBG, and profoundly affects the availability of glucocorticoids to target cells.

(B) Glucocorticoid passage across the cell membrane

The mechanism of glucocorticoid entry into target cells has received relatively little attention. It has been generally assumed that glucocorticoids readily pass across the cell membrane via passive diffusion by virtue of their small size and lipophilic nature. Uptake studies in hepatoma cells (Plageman and Erbe, 1976) and thymocytes (Mayer et al., 1976) have been consistent with this view; however, several systems, such as mouse L-929 and rat liver cells, accumulates glucocorticoids against a concentration gradient (Gross and Aronow, 1970; Rao et al., 1976). In part, such an accumulation against a concentration gradient may be explained by binding to intracellular glucocorticoid receptors, but there seems to be an additional uptake which may represent either low affinity membrane-binding sites, or a facilitated steroid transport system. At present, the evidence for a general existence of either inward or outward facilitated transport remains inconclusive.

(C) Cytoplasmic receptor binding

A central concept in this model of steroid action is that the glucocorticoid must bind to a specific receptor protein in order to exert its effect in the cell. Within target cells, which can be distinguished from non-target cells by autoradiographic technique, the entering glucocorticoid interacts with cytoplasmic receptor (designated as R_a-R_b in Fig. 3) in a non-covalent manner with high affinity, stereo-specificity and finite capacity (Feigelson et al., 1978) to yield a glucocorticoid-receptor complex.

The idea of receptor dimers was originally formulated for the progesterone receptor by O'Malley's laboratory. However, glucocorticoid (Buller et al., 1976), estrogen and androgen (Narris and Kohler, 1976) binding activity could be eluted from various ion-exchange resins as

pairs of peaks reminiscent of the oviduct progesterone system (O'Malley and Buller, 1976). The two subunits of cytoplasmic receptor have previously been classified as the R_a and R_b proteins on the basis of their different affinities for nuclear constituents. Subunit R_a binds to DNA, but not to chromatin, whereas subunit R_b binds to chromatin, and less readily to DNA. Both proteins have identical hormone-binding specificities and kinetics (O'Malley and Buller, 1976). O'Malley proposed that subunit R_a is truly a regulatory protein which might be expected to alter transcription. Conversely, subunit R_b is merely a specifier protein that carries the R_a protein to the neighborhood of responsive genes and is ineffective in stimulating chromatin transcription.

In the early 1960's, intracellular glucocorticoid-binding components were identified in rat liver using radioactive steroid, but their involvement in hormone action was debatable (King and Mainwaring, 1974). A major breakthrough occurred when binding studies were conducted on isolated cells for which steroid concentrations were known with precision and competition and kinetic experiments could be performed, and recourse made to synthetic glucocorticoids that did not interact significantly with nonreceptor components of the cell. Critical investigations by Munck (Munck and Brinck-Johnsen, 1968) and Schaumburg (Schaumburg and Bojesen, 1968) on rat thymocytes provided the first clear demonstration of glucocorticoid receptors. In these cells, a limited number of sites could be detected that bound steroids with an affinity consistent with their glucocorticoid activity. These findings were extended to glucocorticoid-sensitive cell lines in culture: human HeLa cells (Melnykovych and Bishop, 1969), mouse L-929 fibroblasts (Hackney et al., 1970), and P1798 lymphosarcoma (Kirkpatrick et al., 1971), rat hepatoma tissue culture

(HTC) (Baxter and Tomkins, 1970), and S49 lymphoma cells (Baxter et al., 1971). Mammalian skin apparently contains receptor proteins in both epidermis and dermis (Voorhees, 1977b). Preliminary evidence demonstrated the presence of a single class of high-affinity binding proteins in mouse skin (Slaga et al., 1977) and in primary cultures of epidermal cells (Epstein, 1977; Ponec et al., 1980).

When cells containing bound steroid were fractionated, the bound steroid was found primarily in the nucleus (Baxter and Tomkins, 1970; Baxter et al., 1971). In contrast, when binding was examined in extracts of cells that had not been exposed to the steroid, activity was restricted to the cytosol (Baxter and Tomkins, 1971; Pratt and Ishii, 1972). These observations provided the initial indication that the glucocorticoid receptor system is similar to that of other steroid-responsive systems, in that it possesses cytosol receptors which, upon binding an active steroid, associate with nucleus. The nature of the nuclear binding reaction is discussed elsewhere. The model systems studied in the early seventies included rat HTC cells (Baxter and Tomkins, 1970; Baxter and Tomkins, 1971; Rousseau et al., 1972a), liver (Rousseau et al., 1972a; Beato and Feigelson, 1972; Litwack et al., 1973) and thymocytes (Schaumburg, 1972; Bell and Munck, 1973), mouse L-929 fibroblasts (Pratt and Ishii, 1972), lymphosarcoma P1798 (Kirkpatrick et al., 1972) and pituitary tumors (Watanabe et al., 1973), chick embryo retina (Chader et al., 1972) and cultured mammary cells from rat (Gardner and Wittliff, 1973) and mouse (Shyamala, 1974). These investigations gave some insight into the binding kinetics and physicochemical properties of the receptor sites, and established the involvement of the receptor in glucocorticoid hormone action on firmer ground. For a detailed account of the literature on the subject, comprehensive reviews are available (Pasqualini,

1976 and 1977; O'Malley and Birnbaumer, 1977 and 1978; Baxter and Rousseau, 1979).

Ponec et al. (1980) have attempted to relate cytosol receptor binding in human cultured fibroblasts, to growth influenced by glucocorticoids. Under certain circumstances growth inhibitory effects were lost without binding being affected. It would seem another mechanism, perhaps steroid induction of other controlling factors, may be involved.

The cytosol binding of glucocorticoids constitutes both specific and non-specific binding. Munck and Leung (1977) defined the specificity of binding as the ability to form complexes with molecules of structures that lie within the range of stereochemical configurations associated with a particular hormonal activity. Nonspecificity referred to a relative lack of such selectivity. Therefore, not all glucocorticoid binding is receptor binding, nor all bindings result in glucocorticoid activities. This project is mainly concerned with the determination of "specific binding".

(D) Activation and translocation of glucocorticoid-receptor complex

The glucocorticoid-receptor complex undergoes an "activation" needed for translocation into the nucleus. This activation is believed to involve conformational change of the receptor by mechanisms either of driving the equilibrium between two forms of receptors towards the form of higher affinity to glucocorticoid (Monod et al., 1965), or of "induced fit" model (Koshland and Neet, 1968; Atkinson, 1966). The rate of the activation is highly dependent on temperature (Milgrom et al., 1973; Jensen et al., 1972) and ionic strength (Milgrom et al., 1973). It is now thought that only 40-60% of glucocorticoid-receptor complexes become activated (Higgins et al., 1979).

The translocation of activated glucocorticoid-receptor complex from cytoplasm into nucleus is not deniable, but very little is known about how it actually occurs. The glucocorticoid remains bound to the same receptor protein in both cytoplasmic and nuclear compartments during the translocation process. The passage of the complex across the nuclear membrane is not a rate-limiting step in the nuclear accumulation of the complex (Buller et al., 1975).

(E) Nuclear acceptor binding and gene expression

The translocated glucocorticoid-receptor complex associates with acceptor sites in the chromatin, in an as yet undefined manner to form a ternary chromatin-glucocorticoid-receptor complex. These events lead to subtle alterations in the pattern of gene transcription, and finally, the altered or new mRNA, enzyme or other protein levels affect the hormonally evoked cellular and physiological responses.

However, the process leading to the transcriptional alterations and, further, to changes in cellular physiology is the least understood aspect of the receptor model theory. Some of the problems requiring further exploration and a more precise definition are:

(a) the precise chemical nature of the interaction between glucocorticoid-receptor complex and nuclear components, as for example, whether the binding is ionic, hydrophobic or covalent in nature,

(b) the component of glucocorticoid-receptor complex involved in the nuclear binding, i.e. whether the glucocorticoid or the receptor protein, or both are involved in the interaction,

(c) the nuclear component responsible for the specific acceptor binding, among which DNA, histone and non-histone proteins have been proposed (Feigelson et al., 1978; Wong and Aronow, 1976: O'Malley and Buller, 1977),

(d) the dissociation of glucocorticoid from receptor sites and of glucocorticoid-receptor complex from acceptor sites, as well as the glucocorticoid elimination from the cell,

(e) The mechanism of regulation of gene expression induced by binding of glucocorticoid-receptor complex, especially why only a small percentage of genes are affected,

(f) the correlation of nuclear binding with biological response, and

(g) the unexplained dichotomy of glucocorticoid effects which may be due to differences in cell types, cell metabolic states or their state of differentiation as well as on differences in glucocorticoid concentrations and on the lengths of time of exposure of cells to glucocorticoid.

Reviews on these controversial or insufficient studies are presented further in this survey.

VI. Physicochemical characteristics of glucocorticoid receptor and receptor binding

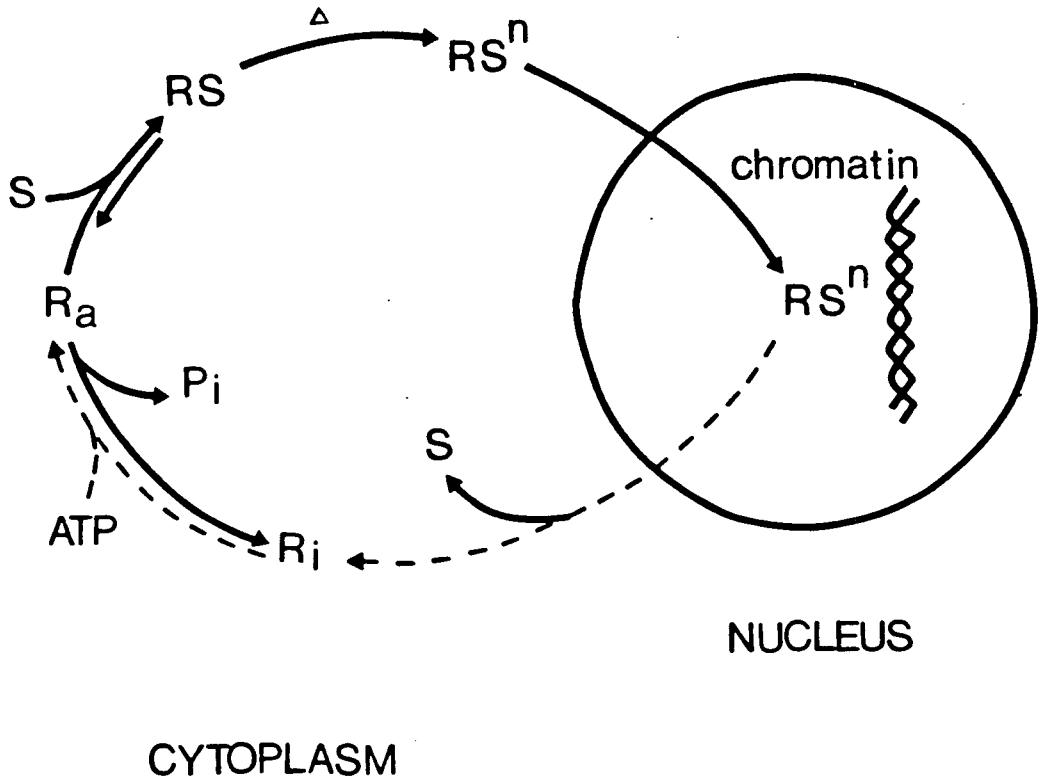
The receptors are proteinaceous in nature, since their binding activity to glucocorticoids is destroyed by pronase, trypsin, papain and chymotrypsin, but not by DNAse, RNAse, collagenase, hyaluronidase, neuraminidase and lysosomal enzymes. Phospholipases A and C devoid of proteolytic activity inactivate receptor binding in mouse L-929 fibroblasts and rat thymocytes, suggesting that phospholipids are important for receptor stability or that the products of the phospholipase reaction denature the receptor (Hackney and Pratt, 1971; Schulte et al., 1976). The amphoteric character of receptor protein is revealed by its ability

to bind to both anionic (phosphocellulose, DNA) and cationic (diethylaminoethylcellulose) polymers.

Glucocorticoid receptors are ubiquitously distributed as physiologic regulators in mammalian tissues (Rousseau and Baxter, 1979). The distribution of glucocorticoid receptors in mammalian tissues has been compiled by Munck and Leung (1977).

It has been proposed that the receptor is cycled through different binding states and locations in the cell (Middlebrook et al., 1975; Munck et al., 1972; Ishii et al., 1972; Nielsen et al., 1977). A single receptor protein may be actually participating several times in the process of steroid binding and gene activation. A model of the receptor cycle proposed by Nielsen et al. (1977) is presented in Figure 4. The cycling of the receptor in its bound form from cytoplasm to nucleus is regarded as followed by regeneration of unbound receptor into the cytoplasm. The receptor cycle may have an important role in determining the ability of the cell to change rapidly the intensity of its physiological response in response to changing plasma glucocorticoid levels (Pratt, 1978).

The binding parameters, the equilibrium dissociation constant, K_D, and the binding capacity, B_{max}, of glucocorticoids have been determined using Scatchard analysis by a number of investigators. Most of the studies have been carried out with dexamethasone and to a lesser extent with hydrocortisone and triamcinolone acetonide in various rat or mouse tissues. Only a few studies have involved other glucocorticoids or human cells. The data summarized in Table III represent the studies reporting the apparent equilibrium dissociation constants of glucocorticoid receptor binding done in the past decade. Variations among the values obtained are large, but most fall within one order of magnitude of



R_i	:	dephosphorylated, inactive receptor
R_a	:	phosphorylated receptor
RS	:	steroid-receptor complex
RS^n	:	activated steroid-receptor complex
S	:	steroid
...	:	processes that appear to require energy in L-cells

Figure 4 : Proposed cycle of events controlling the binding state and cellular location of glucocorticoid receptor (Nielsen et al., 1977)

Table III Binding parameters of naturally occurring and synthetic glucocorticoids to receptors

Glucocorticoid	Tissue/cell line	K_D(nM)	B_{max}	Reference
Hydrocortisone	Mouse L-929 fb.[a]	2.8	-	Pratt et al.,1975
	Mouse L-929 fb.	4.3	1[b]	Middlebrook and Aronow, 1977
	Rat HTC	11	0.63[c]	Rousseau et al., 1972a
Triamcinolone acetonide	Mouse L-929 fb.	1.6	-	Pratt et al.,1975
	Mouse L-929 fb.	0.06	1[b]	Middlebrook and Aronow, 1977
	Human skin fb.	0.4-8.6	1.5-11.4[d]	Bruning et al., 1979
Dexamethasone	Mouse L-929 fb.	7.6	-	Pratt et al.,1975
	Human skin fb.	4.5	0.12[c]	Bauknecht, 1977
	Human amniotic fluid cells	19	0.375[c]	Bauknecht,1977
	Rat HTC	3.1	0.66[c]	Rousseau et al.,1972a
	Rat thymocytes	1.9	-	Schaumburg,1972
	Rat liver	3.7	-	Koblinski et al.,1972
	Rat kidney	8.4	0.31[c]	Rafestin-Oblin et al., 1977
	Rat adipose tissue	6	0.2[c]	Feldman and Loose,1977
	Rat testis	3 ± 2	0.2 ± 0.05[c]	Evain et al.,1976
	Mouse thymocytes	4	10[e]	Duval et al.,1976
	Mouse lymphoma	20	-	Rousseau et al.,1972b
	Rabbit lung cells			Ballard,1977
	freshly isolated	6.9	7500[f]	
	cultured 1-2 weeks	7.9	26500[f]	
	cultured,fetal	10.2	13150[f]	

(a) fibroblasts
(b) nM, (c) fmol/µg protein, (d) fmol/µg DNA, (e) fmol/10^6 cells,
(f) sites/cell

each other. Interpretation of the binding capacity, B_{max}, is made uncertain by inconsistencies in the manner of data presentation which by various authors are expressed variously as fmol per µg protein, fmol per µg DNA, fmol per 10^6 cells or the number of binding sites per cell.

Studies of binding kinetics have confirmed that the glucocorticoid-receptor association is second order and dissociation is first order (Baxter and Tomkins, 1971). The rate of association to the receptor has been found to be slower for glucocorticoids with high affinity than for those with low affinity (Rousseau et al., 1972a; Pratt et al., 1975; Ballard et al., 1975; Kaine et al., 1975). Glucocorticoids that bind with high affinity have been found to have a slow rate of dissociation. For instance, the potent hydrocortisone analog, triamcinolone acetonide, binds exceedingly slowly and has extremely high affinity due to the fact that, once bound, it shows virtually no dissociation during the time period of observation (Pratt and Ishii, 1972; Watanabe et al., 1973; Goral and Wittliff, 1975).

VII. Nuclear binding of glucocorticoids

It is now generally recognized that although cytosol glucocorticoid receptor binding is a necessary step, the nuclear interactions of the complex play the central role of many aspects of steroid hormone action. Cell fractionation (Rousseau et al., 1973; Jensen et al., 1971; Shyamala and Gorski, 1969; Wira and Munck, 1974; O'Malley et al., 1971) and whole cell autoradiographic experiments (Bogoroch, 1969) have shown that radiolabelled steroid hormones are retained and concentrated in the nuclei of target cells of all tissues of the mammalian body. Removal of the cell nucleus, e.g. with cytochalasin B, prevents glucocorticoids from inducing tyrosine aminotransferase in hepatoma (HTC) cells (Ivarie et

al., 1975). The removal of glucocorticoid from cell nuclei by washing stops initiation of transcription in rat pituitary tumor cells (Johnson et al., 1979b).

Glucocorticoids become specifically bound to the nucleus only after binding of glucocorticoid to cytosol receptors. Nuclei isolated from untreated cells do not bind glucocorticoids (Higgins et al., 1973a).

The kinetics of nuclear binding have been studied in HTC cells, rat thymocytes and mouse L-929 cells. In HTC cells exposed to dexamethasone at 37°, nuclear binding of steroid reaches a maximum within 30 min and levels off (Figure 5). The plateau persists as long as the steroid is present (Rousseau et al., 1973). With rat thymocytes, there is a lag period of less than one minute before dexamethasone-receptor complex starts to appear in the nucleus. The nuclear binding then levels off after 5-10 min (Munck et al., 1979). In other cell lines, the steady state of levels of binding are attained more slowly, but are generally reached within approximately 2 hr (Middlebrook et al., 1975).

The majority of investigators whose work dominates the current views on glucocorticoid mechanism of actions, such as Baxter and Rousseau (1979), Pratt (1978), Aronow (1979), Munck and Leung (1977), and O'Malley and Birnbaumer (1977, 1978) emphasize in their experimental design the 0-2 hr, sometimes 0-6 hr time period for studies. They have assumed that after nuclear binding has reached the steady state the glucocorticoid-receptor complex has no further influence on the cellular metabolism. Their assumption should be tested by following glucocorticoid influence through at least two cell cycle periods (about 96 hr for human fibroblasts, 48 hr for L-929 fibroblasts).

The nuclear binding of glucocorticoids in HTC cells has been found rapidly reversible after removal of the steroid from the incubation

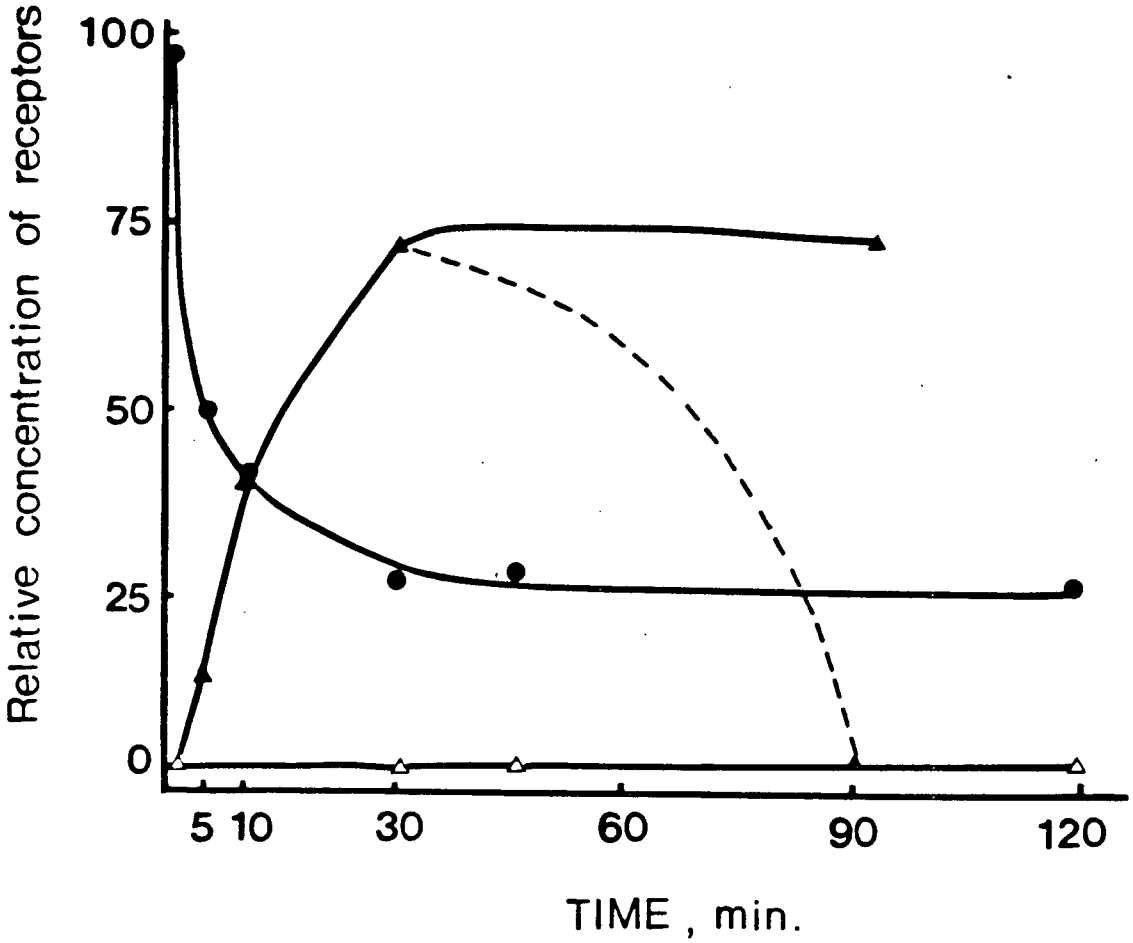


Figure 5 : The kinetics and reversible effects of nuclear binding of dexamethasone-receptor complexes in intact HTC cells. Cytosol (circles) and nuclear (triangles) content of receptor is presented as a function of time following addition of dexamethasone (● , ▲) or of the vehicle (△) to intact HTC cells. Dashed line corresponds to data obtained following removal of steroid from the culture at 30 min. (Rousseau, 1975)

medium (Figure 5). The disappearance of the steroid from the nucleus has been observed to be complete within 1 hr at 37° (Rousseau, 1975).

The nuclear binding was believed initially to be a saturable process with high affinity. However, Chamness et al. (1974) and Milgrom et al. (1975, 1976) and Higgins et al. (1979) have demonstrated that apparent saturation of nuclear sites is due to competition of excess nonreceptor components with specific binding sites. In the presence of a constant concentration of nonreceptor protein, nuclear binding is found to be a nonsaturable linear function of the concentration of the complex. Higgins et al. (1973c) have also suggested that saturable binding in vitro is artifactual by demonstrating that nuclei from cells pretreated with steroid in vivo are still capable of subsequent binding of the complex in vitro. Hence, most laboratories now appear to agree that nuclear binding of complexes is not readily saturable. Lacking saturation, the Scatchard plot for nuclear binding has a slope of zero (Figure 6), and binding affinity and capacity can no longer be estimated by this analysis as it can be done for cytosol receptor binding (Yamamoto and Albert, 1976; Higgins et al., 1979). Lack of saturation can be illustrated by Figure 6 which represents the data of Higgins et al. on dexamethasone-receptor accumulation in HTC cell nuclei.

VIII. Nuclear components associated with glucocorticoid binding

The nucleus plays an important role in the mechanism of glucocorticoid action. Both the accumulation of glucocorticoid-receptor complex and the modification of gene expression occur in nuclei of target cells. In order to discuss the possible mechanism of these two events, a brief review of the nuclear structure is in order.

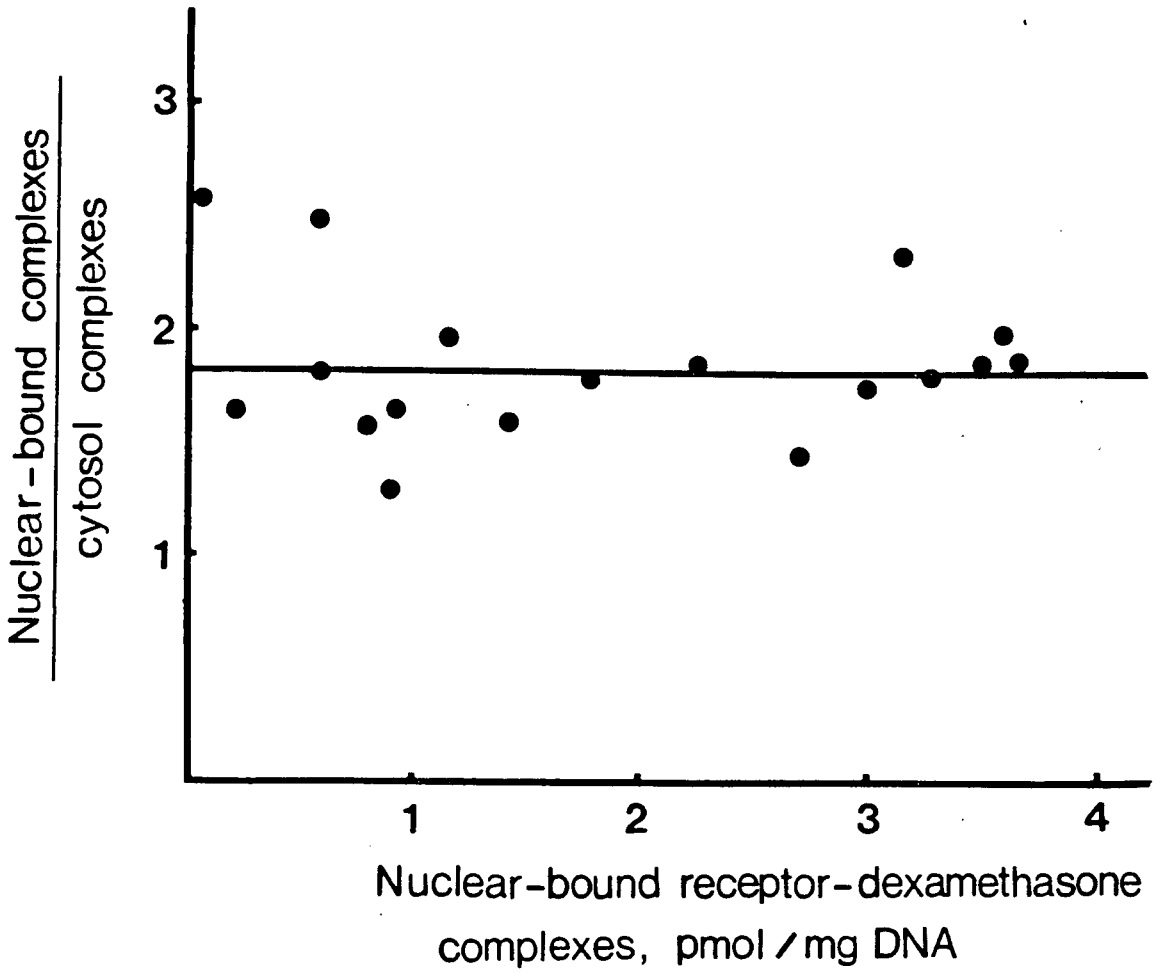


Figure 6 : Independence of nuclear binding of concentration of receptor-dexamethasone complex in the cytoplasm of intact HTC cells at 37^{o}.

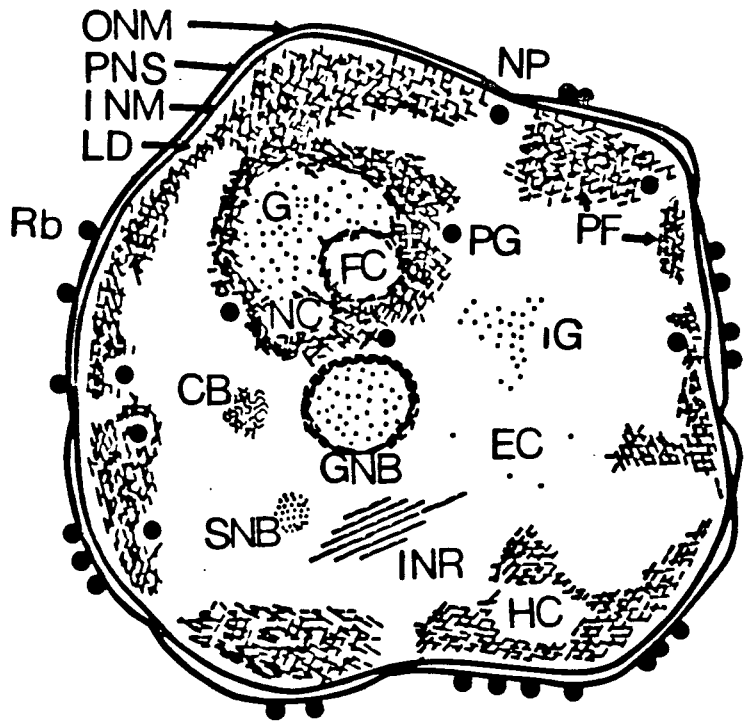
Shown is a Scatchard plot of the data where concentration of the total cytosol receptor-dexamethasone complexes and nuclear-bound complexes were measured.

(Higgins et al., 1979)

The nucleus (Figure 7) is surrounded by the outer (ONM) and inner nuclear (INM) semipermeable membranes that enclose the perinuclear space (PNS) which is a part of the rough endoplasmic reticulum and has ribosomes (Rb) attached. Between the chromatin and the inner membrane lies the lamina densa (LD) which is thinner in front of the nuclear pores (NP). The chromatin is found as heterochromatin (HC), nucleolus-associated chromatin (NC), and euchromatin (EC). The nucleolus shows the granular (G) components and fibrillar centers (FC). In the borderline of the chromatin, many perichromatin granules (PG) and a layer of perichromatin fibrils (PF) are found. Finally, in the interchromatin space, a cluster of interchromatin granules (IG), granular nuclear body (GNB), a simple nuclear body (SNB), a coiled body (CB), and an intranuclear rodlet (INR) are seen.

Busch (1979) tabulated the detailed nuclear structures in terms of nuclear envelope, chromatin, nucleolus, nuclear RNP network and nuclear particles. The nucleus is part of an integrated system by which cells of various organs respond to extracellular and intracellular stimuli. These stimuli interact with nuclear informational systems to produce specific products that permit response of the cytoplasm to the environments and its functional demands.

The nucleus contains the major repository of genetic information, chromatin, which comprises DNA and its associated proteins. When intact cells are exposed to glucocorticoids and the nuclei are then fractionated, glucocorticoid-receptor complexes are found in chromatin but not in the nucleolus, nucleoplasm, or nuclear membranes (Higgins et al., 1979). A similar distribution is seen in liver nuclei after binding of glucocorticoid-receptors <u>in vitro</u> (Beato et al., 1973). In chromatin, nuclear-bound receptor-glucocorticoid complexes are found extensively



Nuclear envelope
- ONM outer nuclear membrane
- INM inner nuclear membrane
- PNS perinuclear space
- NP nuclear pores
- Rb ribosomes

Chromatin-chromosomes
- HC heterochromatin
- EC euchromatin
- NC nucleolus-associated chromatin

Nuclear particles
- PG perichromatin granules
- PF perichromatin fibrils
- IG interchromatin granules
- GNB granular nuclear body
- SNB simple nuclear body
- CB coiled body
- INR intranuclear rodlet
- LD lamina densa
- ^{m}RNP precursor particles

The nucleolus
- nucleoli in various stages of cell function
- G granular components
- FC fibrillar centers
- Pre-rRNA
- Interlocks of rRNA and ribosomal protein synthesis
- The nucleolar channel system

Figure 7: Idealized section of a nucleus, showing all the main components (Bouteille et al., 1974)

distributed in both the transcriptionally active and inactive fractions (Levy and Baxter, 1976).

Glucocorticoid-receptor complexes do not bind to RNA (Rousseau et al., 1975). The evidence for this comes from cell-free systems. In these systems, cytosol receptor is bound with ^{3}H-dexamethasone, then activated by heating to 20^{o} for 30 minutes and incubated with either native HTC DNA or E. coli rRNA. Fractionation through a Sepharose 4B column finds the glucocorticoid-receptor complexes bound with DNA but not with RNA (Figure 8).

The precise nature of glucocorticoid-receptor binding to nuclear acceptor sites in chromatin is still a matter of dispute. Different groups of investigators have various views.

Feigelson et al. (1978) suggested DNA is the major binding component of the glucocorticoid-receptor complex at acceptor sites. He has demonstrated that brief treatment of rat liver nuclei with DNAse I caused a loss of about 10% of nuclear DNA, but resulted in more than 80% of loss in nuclear binding capacity for glucocorticoids. Thus, it appears that glucocorticoid-receptor complex is bound to a small portion of the genome, which is readily sensitive to DNAse. Baxter et al. (1972) and Higgins et al. (1973b) reported similar observations to confirm the involvement of DNA in acceptor binding sites.

O'Malley and Buller (1977) proposed that non-histone proteins play an essential role in nuclear binding of steroids and would explain the tissue specificity of target cells for glucocorticoids. Non-histone proteins, unlike histone proteins, are quite diverse from cell to cell within a species or among species. It has been demonstrated that specific binding of steroid to chromatin can be transferred from a target tissue to a non-target tissue merely by switching subfractions of the

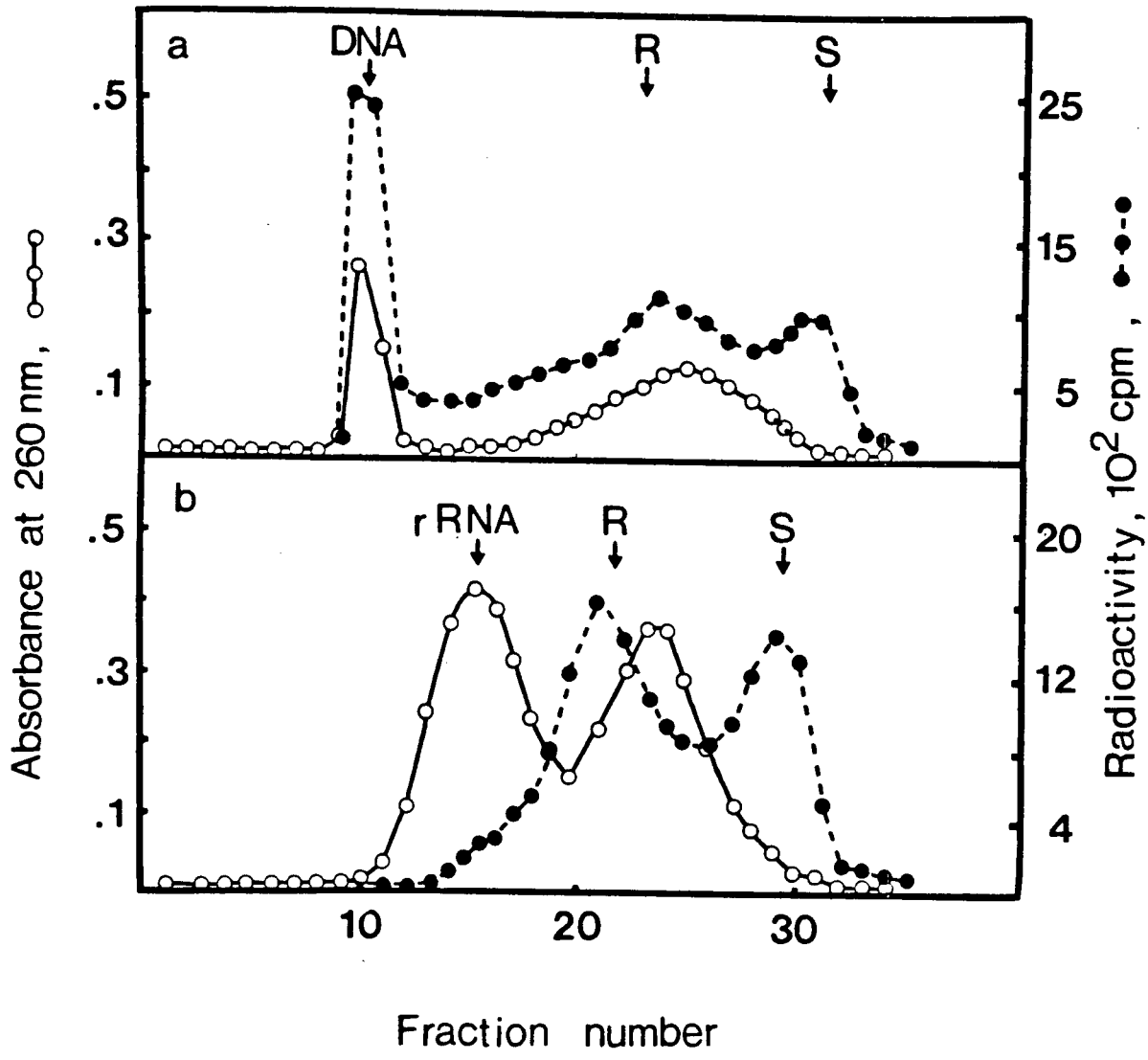


Figure 8 : Glucocorticoid receptors bind to DNA but not to RNA

R: free receptor-steroid complexes

S: free steroid

(Rousseau et al., 1975)

non-histone proteins (Spelsberg, 1975). A major class of non-histone, acidic proteins from oviduct, AP_3, has been shown to contain such a target-tissue specific activity (O'Malley et al., 1972). Puca et al. (1974) and Mainwaring et al. (1976) on the other hand proposed that basic rather than acidic non-histone proteins were responsible for the tissue specificity of nuclear binding.

Wong and Aronow (1976) detected an alteration in the pattern of H_1 histone proteins in L-929 fibroblasts following exposure to glucocorticoids. They thought that glucocorticoid-receptor complexes bind to chromatin by either displacing or modifying H_1 histone proteins.

Direct binding of the glucocorticoid-receptor complexes to DNA occurs in cell-free systems and has been alluded to before. Its occurrence is a definite although inadequately investigated possibility.

IX. Drug interactions with DNA

During the past few years, important advances have taken place in understanding the molecular basis of drug-DNA interactions. These advances include the use of the technique of X-ray crystallography in determining the three-dimensional structures of a large number of drug-nucleic acid crystalline complexes. Types of drug-DNA interactions include intercalation, hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions. Among these interactions, intercalation has received the most attention, because it is believed to be involved in the mechanisms of point mutation, carcinogenesis, cytotoxicity and anticancer activity of drugs. The mechanism is believed to involve damage to DNA by virtue of single strand breaks, double strand breaks, interstrand cross-links and DNA-protein cross-links. The charac-

teristics of such drug-induced macromolecular damage of nuclear DNA are depicted in Figure 9.

Intercalation of drugs into DNA is defined as the insertion of drug molecules between two successive bases in DNA, and it is also termed base-stacking interaction (Weeks et al., 1975). Intercalation is a type of strong interaction between drug molecule and DNA. Known intercalating agents are the bifunctional echinomycin, bisacridine, bisethidium, mixed ethidium-acridine (Wakilin and Waring, 1976; Ughetto and Waring, 1977; LePacq et al., 1975; Butour et al., 1978; Dervan and Becker, 1978), actinomycin (Sobell and Jain, 1972; Sobell et al., 1977b), ethidium (Jain et al., 1977), aminoacridine (Sakore et al., 1977 and 1979; Reddy et al., 1979), ellipticine, tetramethyl-N-methylphenanthrolinium (Jain et al., 1979) irehdiamine (Mahler et al., 1968; Sobell et al., 1977a), psoralen (Cole, 1971; Lown and Sim, 1978; Ley et al., 1977; Wiesehahn and Hearst, 1978) and bleomycin (Umezawa et al., 1973; Muller et al., 1972; Bearden et al., 1977) Besides the steroidal diamine, irehdiamine, these intercalating agents are all planar heterocyclic in structure.

A current concept of Sobell (1979) and others consider intercalation to result from the dynamic nature of DNA conformational changes. According to this view, the DNA molecule undergoes wave-like propagation in its polymeric structure, that is excited through impulses generated at random by the continuous bombardment of DNA by solvent molecules along its length. The wave propagation may involve the motions of bending, stretching, shearing and unwinding, and results in structural distortion of DNA. At some critical oscillation amplitude, alternate sugars "snap into" a C_3' endo sugar conformation with a concomitant partial unstacking of base pairs. Such structure is denoted β-kinked DNA (Figure 10) and is favorable for drug intercalation (Figure 11). A simi-

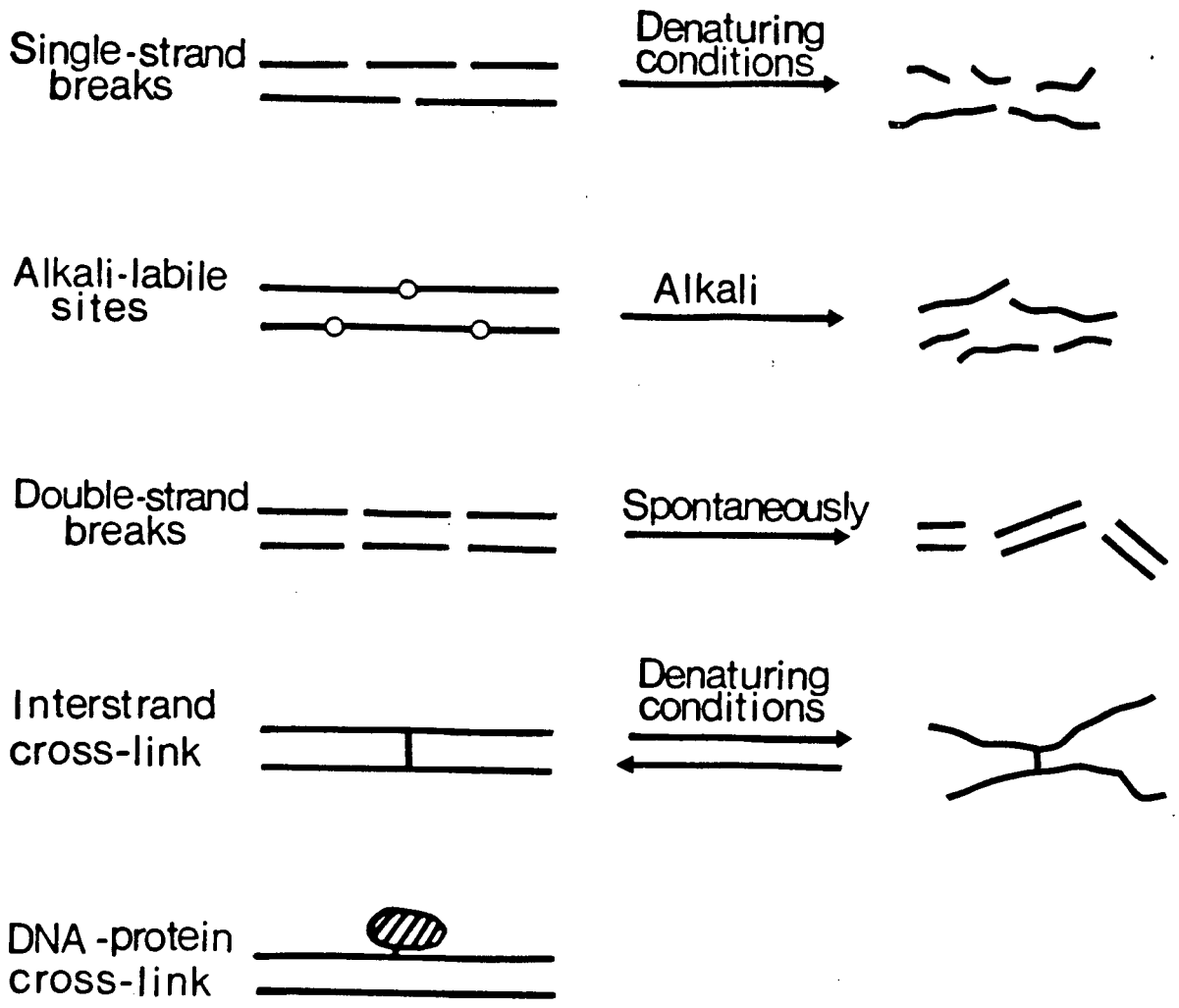
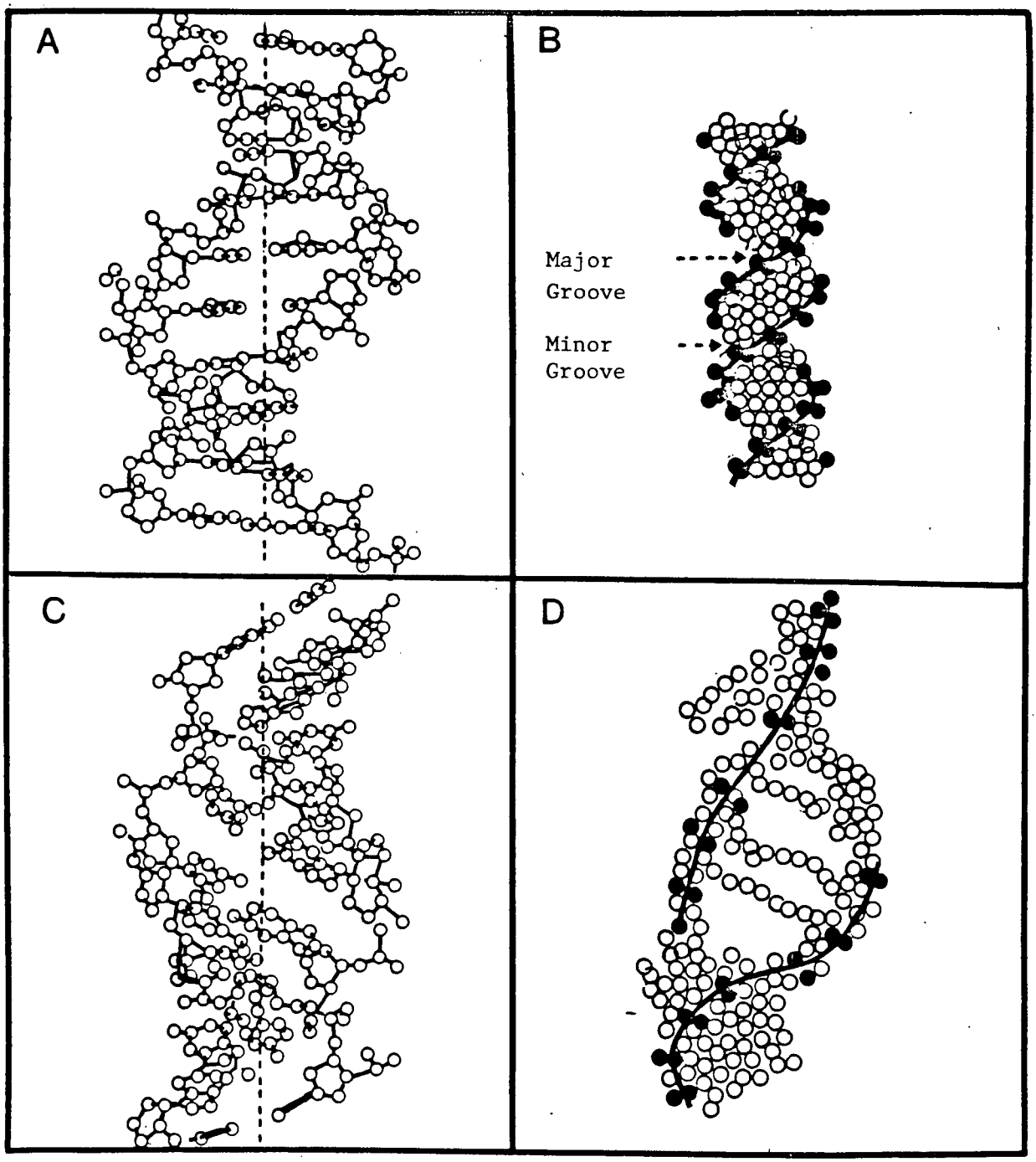


Figure 9 : Drug-induced macromolecular damage of nuclear DNA (Kohn, 1979)



(A) B DNA drawn by computer graphics

(B) B DNA, Corey-Pauling-Koltun (CPK) space filling model

(C) β-kinked DNA, drawn by computer graphics

(D) β-kinked DNA, CPK space filling model

Figure 10 : DNA undergoing wave-like distortion in its structure (Sobell, 1979)

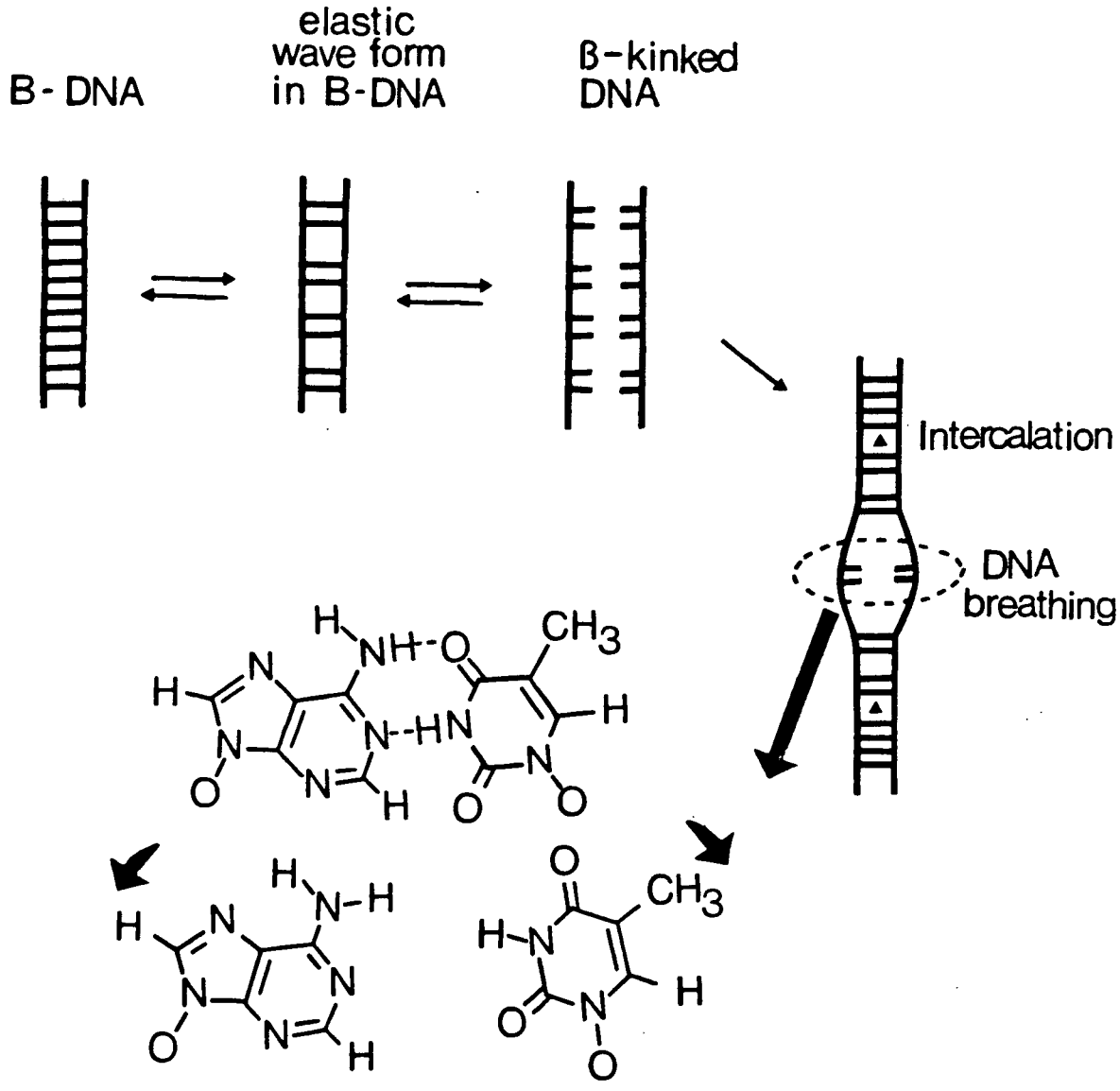


Figure 11 : Schematic illustration of dynamic concepts of DNA structure involved in DNA breathing and in drug intercalation

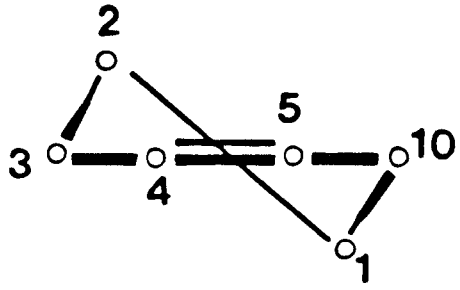
(Sobell, 1979)

lar ß-kinked conformation is proposed as a key intermediate in DNA unwinding for RNA synthesis in vivo.

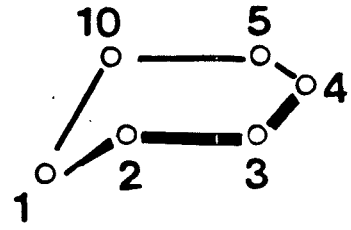
X. Glucocorticoids as potential intercalators

Glucocorticoids and other Δ^4-3-one A-ring containing steroids, such as progesterone and deoxycorticosterone, have been proposed by Duax et al., (1976) as potential nucleic acid intercalators, based on their studies on the structural X-ray analysis of deoxycorticosterone (DOC)-adenine crystalline complex.

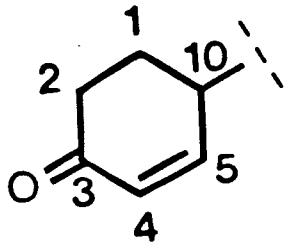
The conformation of Δ^4-3-one A-ring was found between the 1α-sofa and 1α, 2ß-half chair forms (Figure 12) from crystallographic analysis (Duax and Norton, 1975). In the optimized molecules, the ring was consistently closer to the 1α, 2ß-half chair conformation (Schmit and Rousseau, 1978; Duax et al., 1976), and was very planar (Duax et al., 1976). In the crystal structure of DOC-adenine complex, two kinds of interactions, hydrogen bonds (Figure 13) and steroid-base stacking (Figure 14), have been observed. Weeks et al. (1975) suggested that although the nature of the interactions between the hormone-receptor complex and the DNA molecule is unclear, the mere fact that a stable DOC-adenine crystalline complex can be formed is interesting and possibly significant. They also considered the hydrogen bond pairing between the corticoid side chain and the adenine unlikely to be a biologically significant initial reaction between a corticosteroid and DNA since the adenine atoms involved in the hydrogen bonds normally participate in base pairing interactions. However, this corticoid-base pairing could stabilize a DNA conformation in which a break had already been made. On the other hand, the fact that adenine stacks over the unsaturated DOC A-ring, in preference to forming stacks of adenine molecules alone, indicates



1α, 2β-half chair



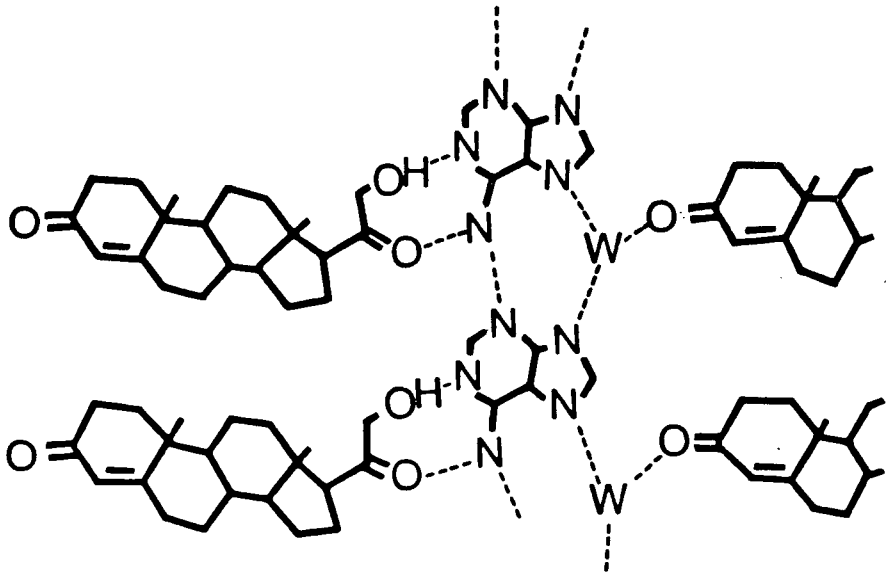
1α- sofa



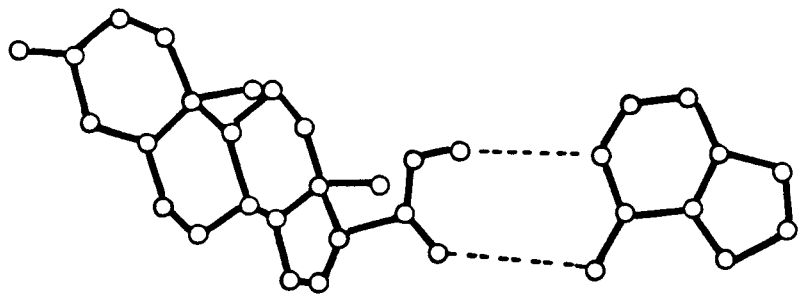
Δ^4-3-one A ring

Figure 12 : Conformations of Δ^4 - 3 - one A ring

(Schmit and Rousseau, 1979)

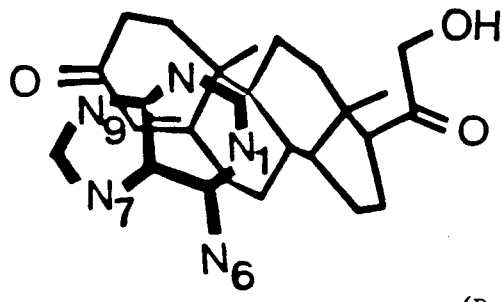


(Weeks et al., 1975)

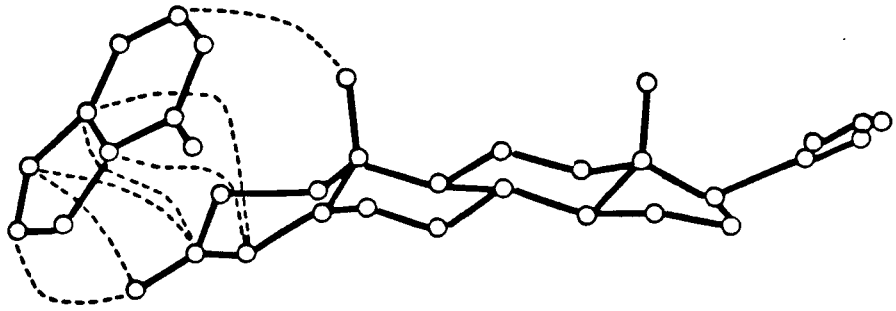


(Duax et al., 1976)

Figure 13 : Hydrogen bonding in the DOC-adenine complex viewed perpendicular to the adenine plane



(Duax et al., 1976)



(Weeks et al., 1975)

Figure 14 : Stacking of adenine and the Δ^4 - 3 - one region of DOC

that it may not be unreasonable to regard the Δ^4-3-one A-rings of progesterone and the corticoids, including glucocorticoids, as potential intercalators.

There is no evidence that direct interaction of DNA with glucocorticoids precedes the appearance of any glucocorticoid-induced effect. The presence of a glucocorticoid-receptor complex in nuclei is needed to elicit the response. Many investigators have reported interactions of glucocorticoid-receptor complexes with DNA in cell-free systems: Steroids have been shown to bind to purified native and denatured DNA (Cohen and Kidson, 1970; Arya and Yang, 1975) and to protect the DNA secondary structure from thermal denaturation (Arya and Yang, 1975). Similar observations were reported by Milgrom et al. (1976), Atger and Milgrom (1978) and Kidson et al. (1970). Glucocorticoid-receptor complexes were found to interact with chromatin (O'Malley et al., 1977; O'Malley and Buller, 1976; King and Gordon, 1972; Baxter et al., 1972; Cake et al., 1978; Duncan and Duncan, 1973; Kalimi et al., 1975).

Duax et al. (1976) proposed that, at the time of interaction of the steroid-receptor complex with the DNA, the steroid may be at the interface between the protein and the DNA. The protein may initiate uncoupling of DNA and the steroid interaction stabilize it, or vice versa (Duax et al., 1976). Moreover, O'Malley has recently demonstrated that in the case of progesterone, the receptor is a dimer binding two steroids, one of which interacts with non-histone chromosomal protein and the other with nucleotide residue of DNA (Schrader et al., 1975).

XI. Nuclear residual forms of glucocorticoid-receptor complexes

Studies on subcellular distribution of ^{3}H-TA in mouse L-929 fibroblasts have revealed a fraction of bound glucocorticoid in the

nuclear pellet that is resistant to extraction with 0.3 M KCl (Middlebrook et al., 1975). This fraction, designated as "nuclear residual form" of glucocorticoid-receptor complex, was believed to be tightly bound to chromatin. A similar residual nuclear binding has also been described in dexamethasone-treated HTC cells (Higgins et al., 1973c). As shown in Figure 15, not more than 80% of dexamethasone-receptor complexes bound to HTC nuclei could be extracted by up to 0.5 M NaCl in intact cells or cell-free experiments. The existence of such "nuclear residual forms" would imply strong glucocorticoid binding to the chromatin, perhaps to the DNA, which might be preserved during isolation procedures of DNA.

Therefore, it was considered worthwhile to test the hypothesis that, after the nuclear binding of glucocorticoid-receptor complex to chromatin, the glucocorticoid molecule might become intercalated into the DNA helix at some point of time during the metabolic cycle of the cell as proposed but not further studied by Duax et al. (1976) for DOC-adenine complexes. Such intercalated glucocorticoids would be tightly bound to chromatin, and might be responsible for the nuclear residual binding of glucocorticoids. When the cultured cells have been incubated with glucocorticoid for a prolonged period of time, it is possible, at certain stages of the cell cycle, to have the required critical β-kinked conformation of DNA structure, favorable for intercalation to occur.

The psoralens, like a large number of other drugs that interact strongly with nucleic acids, are believed to intercalate between base pairs (Wiesehahn and Hearst, 1978; Cole, 1971). Subsequent exposure to long wave-length ultraviolet radiation (320-400 nm) leads to the cross-linking between psoralen double bonds and pyrimidines on opposite strands of a DNA double helix (Cole, 1971; Dall'Acqua et al., 1972). This stabi-

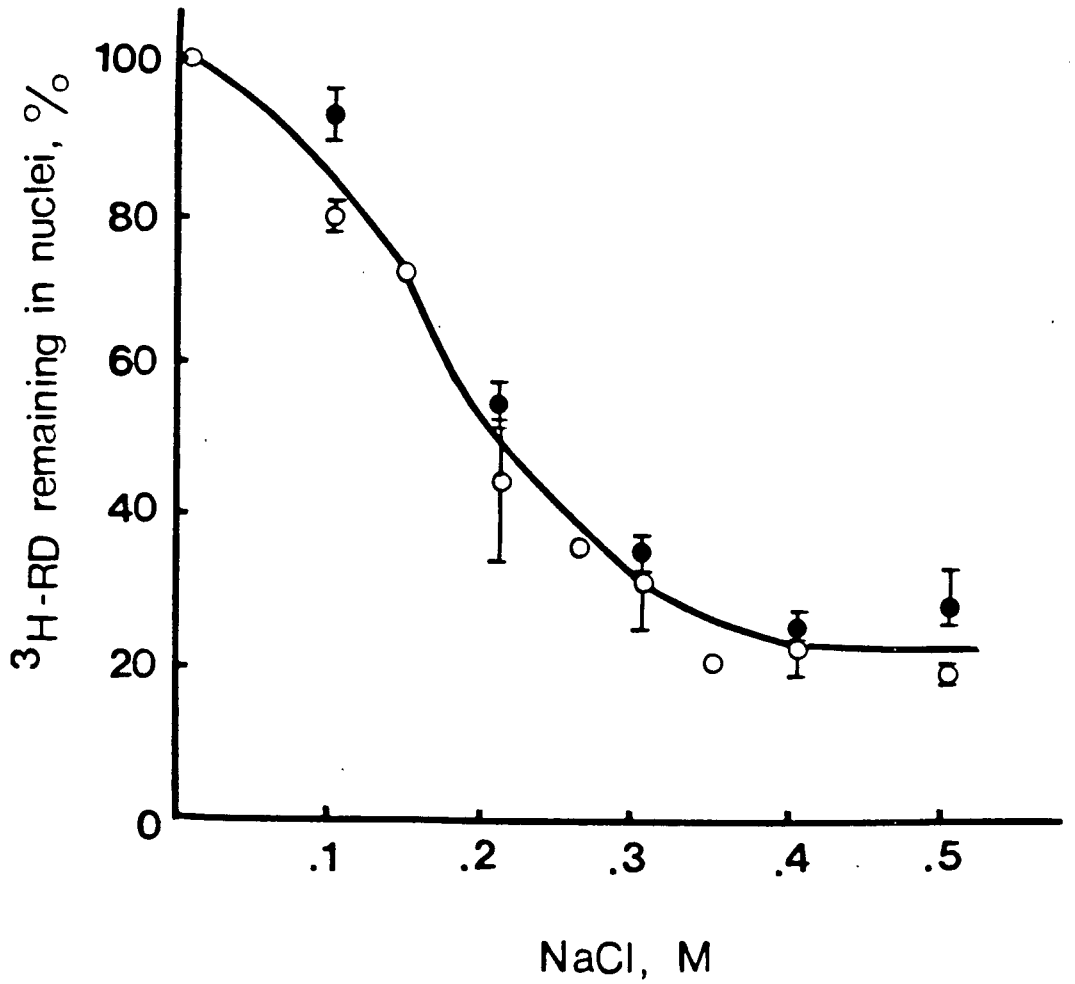


Figure 15 : Release of receptor-^{3}H-dexamethasone complexes (RD) from HTC cells nuclei by NaCl. (Higgins et al., 1973c)
Washed HTC cell nuclei containing ^{3}H-RD complexes bound in intact cell (●) or in a cell-free system (○) were exposed to increasing concentrations of NaCl. After 30 min. at 0°C, the nuclei were washed and assayed for retained complexes

lizes the DNA helix and can be detected by HAP chromatography and denaturation-renaturation analysis. The cross-linking of DNA induced by psoralens with UV irradiation has been established with purified DNA (Pathak et al., 1974; Pathak et al., 1977), in human and mouse tissue culture cells (Cech and Pardue, 1976; Wiesehahn et al., 1977) and in vivo in mammalian skin (Pathak and Kramer, 1969; Dall'Acqua et al., 1972).

Psoralen plus long-wavelength UV irradiation was therefore selected as a model of intercalation for testing whether the presence of intercalation of glucocorticoids into DNA of cultured mouse L-929 and human dermal fibroblasts can be demonstrated. Drugs believed to intercalate DNA include the trypanocide (ethidium bromide), the antitumor antibiotics (daunomycin and adriamycin), the antimalarial drug (chloroquine), the carcinogen (3,4-benzpyrene) and the radical ion of the tranquillizer (chlorpromazine) (Waring, 1968). Each of these drugs binds to DNA by intercalation, but result in diverse biological manifestations. Intercalation is, therefore, a process which provides a strikingly versatile molecular basis for drug action (Waring, 1970). Variations in DNA lesions induced by intercalators are demonstrated in Table IV. Among these agents, psoralen elicits effects on DNA similar to those produced by glucocorticoids. Both are anti-psoriatic agents, and both have antiproliferative effect on dermal cells. The major DNA lesion induced by psoralen plus UV is interstrand cross-linking thought to prevent the strand separation which must occur for normal DNA replication and transcription.

XII. Techniques for detecting cross-links

A number of physicochemical properties of, characteristic of intercalation, DNA have been reported. They include:

Table IV DNA lesions produced in mammalian cells by various agents

Agent	SSB		DSB	ISC	DPC	Base damage
	Direct	Enzymic				
X-rays	++		+	-	+	++
UV light (254 nm)	-	+	-	-	+	++
Bleomycin	++		+	-	-	-
Nitrogen mustards and mitomycin	-			++	++	++
Chloroethylnitroso-ureas (BCNU, CCNU, chlorozotocin)	+			++	++	++
Cis-Pt (II)	-			++	++	++
Trans-Pt (II)	-			±	++	++
Adriamycin and other intercalators	+			-	+	-
Psoralen plus light	-		-	++	±	++

SSB, single-strand breaks; DSB, double-strand breaks;

ISC, interstrand cross-links; DPC, DNA-protein cross-links.

(Kohn, 1979)

(a) increased thermal stability as demonstrated in aminoacridine-DNA complex (Gersch and Jordan, 1965) and in anthracycline antibiotics, daunomycin and nogalamycin, intercalated DNA (Kersten and Kersten, 1968).

(b) increased rate of elution of DNA by alkaline filter method (Kohn, 1978 and 1979) with intercalating agents causing strand breaks and decreased elution rate with those exerting cross-links.

(c) increased viscosity and decreased sedimentation coefficient as observed with acridine or with ethidium intercalated in closed circular ϕ x 174 RF DNA (Kersten et al., 1966; O'Brien et al., 1966; Muller and Crothers, 1968; Waring, 1970), and

(d) shift of visible spectrum as reported in systems of aminoacridines with DNA (Bradley and Wolf, 1959; Peacocke and Skerrett, 1956).

As illustrated in Figure 9, cross-linked DNA behaves differently upon thermal denaturation from regular DNA. Regular DNA responds to thermal denaturation with complete strand separation which results in a kinky supercoiled single-stranded form, when the denatured sample is cooled. On the other hand, the cross-linked DNA demonstrates partial strand separation, but the separated strands are readily reannealed when conditions of helix stability are restored on cooling. Renaturation occurs because the partially separated strands are still held in the vicinity to each by the intercalating molecule. It has been reported by Geiduschek (1961) that a single cross-link in an unnicked, double-stranded DNA molecule is sufficient to hold the complementary strands in register during denaturation and thus leading to rapid renaturation.

(A) Hydroxyapatite chromatography

Hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$, is a complex of $Ca(OH)_2$ and 3 $Ca_3(PO_4)_2$. It was originally developed by Tise-

lius, Hjerten and Levin (1956) for protein chromatography, and was first used with nucleic acids by Semenza (1957) working in Tiselius' laboratory and by Main and Cole (1957). Hydroxyapatite chromatography has been widely used in preparative biochemistry, and has proven itself a versatile technique for the fractionation and purification of proteins (Hjerten, 1959; Tiselius et al., 1956), enzymes (Lindstadt et al., 1970), nucleic acids (Meinke et al., 1974; Coleman et al., 1978), viruses (Smith and Lee, 1978) and other macromolecules (Garola and McGuire, 1977; Trafford et al., 1975; Pavlik and Coulson, 1976; Peck and Clark, 1977).

The basis of rapid isolation of purified DNA from cell lysate by hydroxyapatite chromatography lies in the differential affinities of DNA, RNA, proteins, carbohydrates and various low-molecular-weight substances (Bernardi, 1971a). In principle, the high affinity of double-stranded DNA to hydroxyapatite permits the isolation of DNA, free of contaminants which are less tenaciously bound, simply by loading the tissue lysate onto hydroxyapatite column and then eluting with phosphate buffers of appropriate concentrations (Markov and Ivanov, 1974). The main factor involved in the adsorption of nucleic acids on hydroxyapatite column seems to be the interaction between the phosphate groups of nucleic acids and calcium ions on the surface of hydroxyapatite crystals (Bernardi, 1971a). No significant changes in the physical, chemical and biological properties of native DNA take place upon the adsorption-elution process (Bernardi, 1969a). Native DNA is eluted at 0.20-0.25 M potassium phosphate (KP). The denatured DNA and RNA are eluted at potassium phosphate molarities which are distinctly lower (0.10-0.15 M) (Parish, 1972). Oligonucleotides are eluted at even lower molarities (0.001-0.015 M), and most proteins are adsorbed much less strongly than native DNA. Polysaccharides and small organic molecules are adsorbed very weakly, if at all

(Bernardi, 1969a). Thus, native and denatured DNA can be easily separated by either stepwise or gradient elution.

The reduction in the elution molarity required which occurs when rigid double-stranded molecules are denatured is a special case in the generally observed tendency of more flexible molecules to have lower elution molarities than more rigid ones (Bernardi, 1971a; Martinson, 1973a). It has been proposed that decreased affinity of the more flexible denatured DNA molecule results from an unfavorable configurational entropy change imposed on flexible molecules (Martinson, 1973a). The X-ray diffraction analysis of secondary structures of both native and denatured DNA indicated that the affinity of a rigid, helical nucleic acid for hydroxyapatite is governed by the steric availability of its backbone phosphates for adsorptive interactions (Martinson, 1973b).

The method of the fractionation of chromosomal proteins by the hydroxyapatite dissociation technique was developed by Bloom and Anderson (1978), based on the ability of hydroxyapatite to bind native chromatin in solutions which do not dissociate chromosomal proteins from the DNA. The proteins were then selectively dissociated from the immobilized chromatin by treatment with NaCl, or with NaCl plus urea. The hydroxyapatite dissociation method represented a rapid one-step fractionation procedure which resulted in the quantitative recovery of chromosomal proteins devoid of nucleic acids (Bloom and Anderson, 1978).

The patterns of dissociation of both histone and non-histone chromosomal proteins by NaCl and urea, on the basis of the differential affinities of chromosomal proteins to DNA, were reported by Bloom and Anderson (1978) as presented in Table V.

(B) Thermal scanning analysis

Denaturation of DNA is defined as the transition of double-heli-

Table V Dissociation pattern of histone and nonhistone chromosomal proteins by NaCl and urea from hydroxyapatite column

Fraction	Eluent*			Components of eluate
	NaP(mM)	NaCl(M)	Urea(M)	
A	10	-	-	Unbound chromosomal proteins
B	80	-	-	Unbound chromosomal proteins
C	80	0.25	-	Loosely bound nonhistone proteins (NHP)
D	80	0.5	-	Very lysine-rich histone protein, H_1
E	80	0.75	-	Very lysine-rich histone protein, H_1
F	80	1.25	-	Moderately lysine-rich histone proteins, H_2A & H_2B
G	80	2	-	Arginine-rich histone proteins, H_3 & H_4
H	80	2	2	Tightly bound nonhistone proteins (NHP)
I	80	2	4	Tightly bound nonhistone proteins (NHP)
J	80	2	8	Tightly bound nonhistone proteins (NHP)
K	500	2	5	Nucleic acid (NA)

* All eluents contained 5 mM $NaHSO_3$ to reduce proteolytic activity (Panyim et al., 1968).

cal form of DNA into disordered random coils by disruption of hydrogen bonds between base pairs and of hydrophobic interactions between neighbouring stacked bases (Lehninger, 1975). Denaturation of DNA can occur at extreme pH or at elevated temperature. In the latter case, it is called thermal denaturation. The basic mechanism of denaturation is depicted in Figure 16 .

Based upon both theory and experiment, helix-coil transition in DNA consists of the following key elements:

(a) A nucleation step, which is the denaturation of the first base pair in a helical segment. In this step, both hydrogen bonds in the base pairs on either side of the denatured base pair are destroyed.

(b) A propagation step, which is the breaking of the hydrogen bonds in a base pair next to a denatured (coiled) segment. Only one stacking interaction with the adjacent base pair in the helical segment has to be disrupted in this step; because of this difference in stacking interactions, there is a tendency for a coiled segment to grow instead of generating more short coiled segment which is the basis for cooperative transition when a DNA is denatured.

(c) A strand separation step, which involves a physical separation of both complementary strands of DNA.

(d) Since a G C pair is thermodynamically more stable than an A T pair, more free energy is needed to denature a G C pair than an A T pair (Marmur and Doty, 1962; Crothers et al., 1965; Crothers, 1968; Tinoco et al., 1973).

(e) Electrostatic repulsion between phosphates on the negatively charged phosphate lattice of DNA tends to destabilize the helical structure; charge neutralizaton on phosphates by cations stabilizes greatly the double helical DNA (Marmur and Doty, 1962; Dove and Davidson,

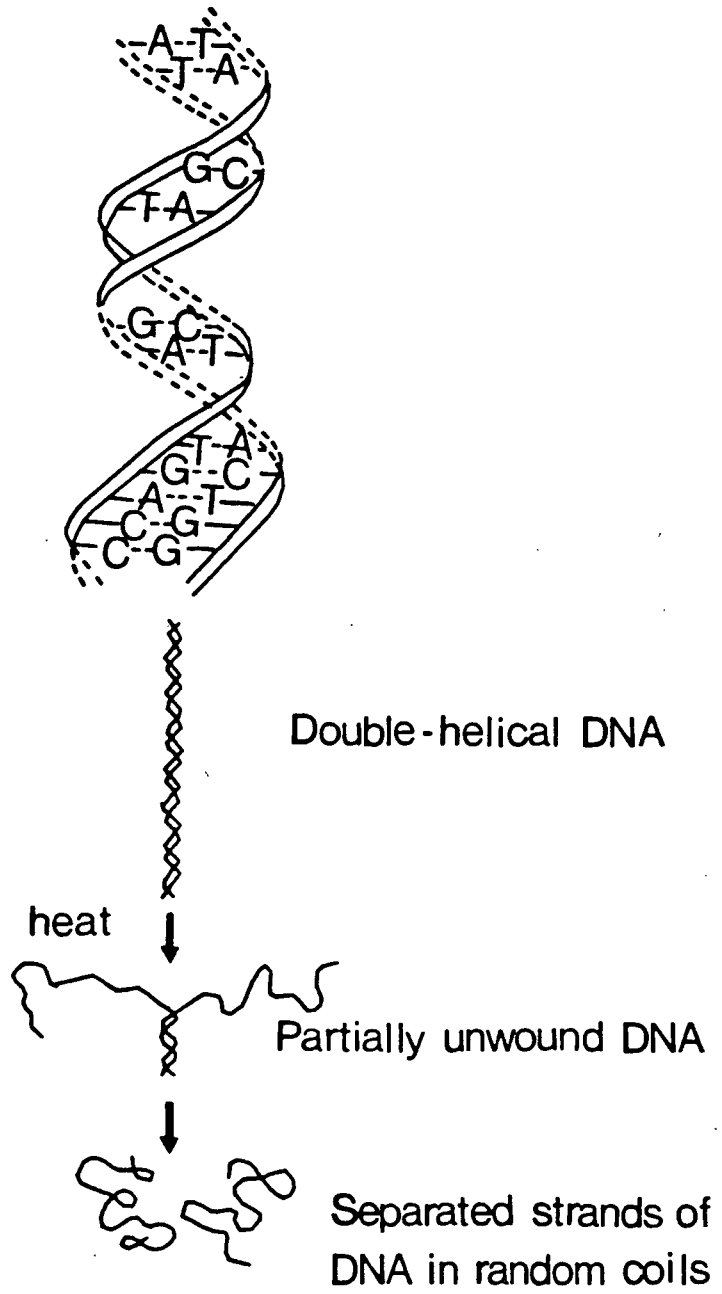


Figure 16 : Basic action of DNA with increasing temperature. The complimentary strands of DNA unwind or denature upon heating. The denaturation or strand separation is followed spectrophotometrically by an increase in absorbance at 260 nm

1962; Schildkraut and Lifson, 1965; Record, 1967; Gruenwedel and Hsu, 1969; Manning, 1972).

Such helix-coil transition in DNA has been discussed in many articles and treated very extensively in the books of Poland and Sheraga (1970) and of Bloomfield, Crothers and Tinoco (1974).

Denaturation of a double-helical DNA is accompanied by an increase in absorbance. This phenomenon is called hyperchromism and is attributed to the destruction of stacking interaction among nucleotides in DNA (Tinoco, 1960; Tinoco, 1961; DeVoe, 1965; Felsenfeld and Hirschman, 1965). The specific effects of elevated temperatures on the DNA absorption spectra are shown in Figure 17. The absorption spectrum of a DNA sample was taken at various temperatures and superimposed for comparison. As the temperature changes gradually from 60° to 80°, there is a sharp increase in absorption centered around 260 nm. Further elevation of temperature shows a subsequent increase to a maximum.

It was observed that denaturation of DNA occurred in a narrow temperature range (Marmur and Doty, 1962). The temperature at which 50% of native DNA is denatured is defined as the melting temperature, T_m, of the DNA. Both T_m and hyperchromicity are parameters defining the thermal denaturation of a given DNA sample.

Thermal scanning technique involves the elevation of temperature up to beyond the melting range with small increments, not greater than 1° per min, and the monitoring of absorbance at 260 nm, A_{260}. The denaturation profile is constructed by plotting the relative absorbance against temperature. The renaturation profile is similarly obtained by reversing the temperature. This technique is of use in characterizing a DNA denaturation-renaturation profile which is sensitive to interactions within DNA helix and interaction between protein and DNA. Therefore,

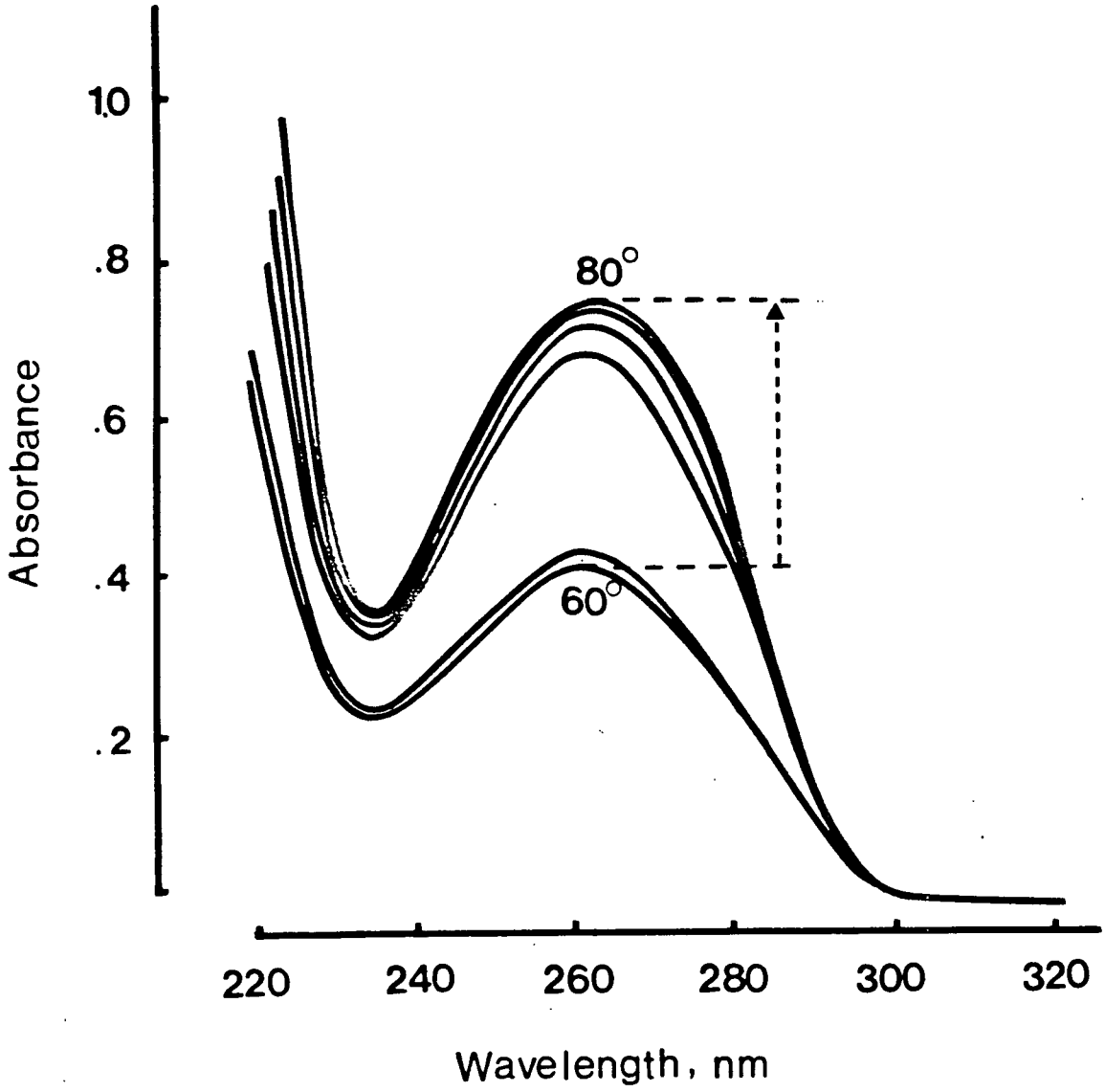


Figure 17 : Hyperchromic effect of DNA upon heating.

Note that absorption increases are centered around 260 nm as the temperature increases from 60^{o} to 80^{0}. This increase (hyperchromicity) stems from thermal denaturation of the double strands of the DNA molecule.

thermal scanning analysis is of potential application in revealing the possible interactions between complex and DNA or between glucocorticoid and DNA. Such applications have been demonstrated by Cech et al. (1979) and Li (1977).

XIII. Regulation of gene expression

The molecular mechanism by which the receptor-genome interaction regulates genetic expression in target cells has been especially scrutinized over the last decade. However, at the present time, even the best data still do not reveal how the steroid-receptor complex is involved in this regulation (Gorski and Gannon, 1976). In the past few years, almost every possible control mechanism involved in gene expression has been advocated, including transcriptional control posttranscriptional control, translational control and changes in enzyme and mRNA stabilities (Feigelson et al., 1978), but Gorski and Gannon (1976) commented that many of the proposed correlations between steroid-receptor nuclear binding and gene expression appeared invalid when closely examined, and confirmed correlations frequently lack general applicability. Moreover, as pointed out by Stormshak et al. (1978), the majority of research efforts have neglected to study in detail the steroid-receptor regulation of DNA replication.

It is recognized that nuclear binding of glucocorticoids can be only one of the triggering steps in the complex sequence of the reaction, leading through transcriptional, and perhaps other DNA-dependent functions, to the great diversity of glucocorticoid tissue-specific responses. Such tissue-specific glucocorticoid actions range, for example, from induction of enzymes, as tyrosine aminotransferase in hepatoma tissue culture (Rousseau et al., 1972a) and proteinaceous hormones, as

growth hormone in cultured pituitary cells (Kohler et al., 1968), inhibition of DNA synthesis by unknown mechanisms, as in human epidermal cells (Voorhees, 1977b) and in mouse L-929 fibroblasts (Pratt and Aronow, 1966) to stimulation of DNA synthesis in various fibroblast cultures (Runikis et al., 1978; Kirk and Mittwoch, 1977; Thrash et al., 1974), and cytolysis of steroid-sensitive lymphocytes (Baxter et al., 1971).

However, these specific but divergent cellular responses of various target cells are thought to originate at the level of the nucleus through modification of gene expression. What remains unclear is what molecules are involved besides the glucocorticoid-receptor complex and whether the reactions involve DNA directly or indirectly (Higgins et al., 1979).

A number of models for reactions involving gene expression have been suggested. Thompson et al. (1977) described a model of negative or positive controls as depicted in Figure 18. The repressor model of negative control supposes that the steroid-receptor complex inherently possesses a high affinity for appropriate gene sites. An example of such negative control might be a histone or other protein, which block genes that would otherwise have high affinity for the steroid-receptor complex.

The adapter model of positive control supposes that some molecule intervenes between the hormone-receptor complex and the genes it affects. This molecule would bind to the complex and thereby confer upon it cistron specificity. An array of such control molecules would be required to provide specific linkage to the variety of specific genes steroids affect. The question of how differentiated cells determine which control molecules to produce is not addressed by these models. A number of experiments have been carried out trying to identify such molecules, and candidates have been found in both the histone and non-histone

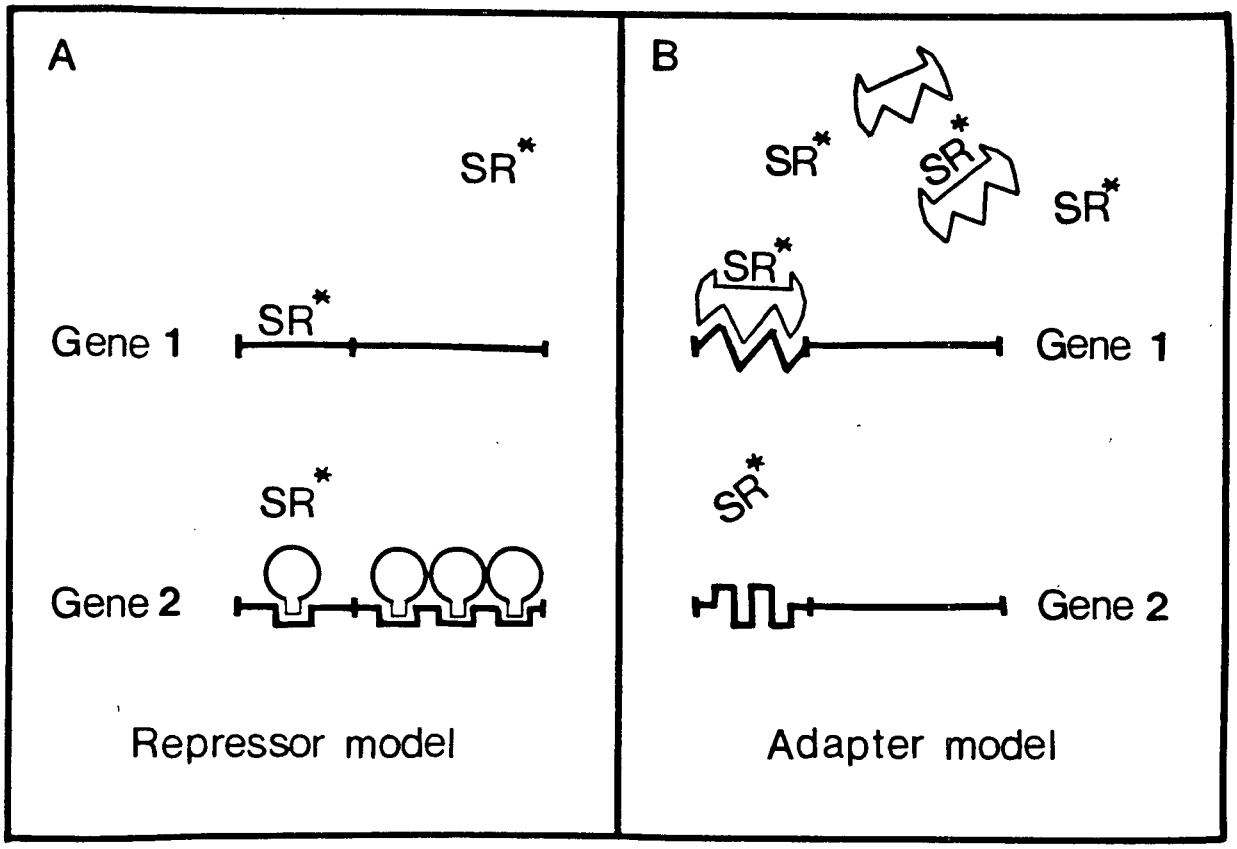


Figure 18 : Models for two types of possible intranuclear control over steroid-receptor complex interactions with DNA

(A) A pure negative-control system, with specifically designed proteins blocking access of activated complex (SR*) by binding to one gene, while leaving open another for interaction with SR*.

(B) A pure positive-control model, in which activated steroid receptor (SR*) in the nucleus binds to a specific site on an intermediary acceptor molecule, which in turn specifies, by virtue of its second, gene-specific site, the gene to which the ternary complex will bind.

fractions of the nuclear proteins (O'Malley et al., 1972; Spelsburg et al., 1972; Puca et al., 1974). Of course, small molecules could be part of the controls operative in either model (Cake et al., 1976).

This positive control model predicts that cells differentiate by losing or failing to make the intermediary adapter molecules. In a cell with functional steroid receptors, the choice of specifically induced products, whether few or many, would depend entirely on the presence of the positive adapters. Overlapping models are possible.

Johnson et al. (1979a) proposed "stoichiometric" or "catalytic" models. The stoichiometric model assumes that receptor-steroid complexes bind specifically at certain sites on chromatin to regulate transcription of specific mRNAs. The site-specific localization of the glucocorticoid-receptor complex would be due either to specific sequences on DNA that bind the complexes, or to chromatin proteins located at specific sites that bind or influence the binding of complexes. The catalytic model assumes that the complex either activates an enzyme, or in some other ways modify molecules important for gene expression. Yamamoto and Alberts (1976) hypothesized that specific hormone effects required clustered receptor complex binding, since only this led, in their view, to formation of a large enough patch to include both a specific promotor and its structural gene.

The specificity of the glucocorticoid responses may exist as controls on the accessibility of specific genes for binding, by virtue of cell differentiation, as proposed in repressor and adaptor models. Or, it may be at levels distal to the receptor-acceptor interaction that only the trigger of modified gene expression is modulated as suggested in stoichiometric and catalytic models. None of the models as yet, have been convincingly defined in biochemical terms. However, it is generally

agreed by Thompson et al. (1977) and Johnson et al. (1979a) that binding of receptor-steroid complex to most of acceptor sites does not elicit biologically relevant responses. Only a few of these acceptors, with affinity same as or higher than most of the detectable acceptors, are located at sites where the expression of specific genes can be modified.

XIV. Dichotomy and time profiles of glucocorticoid effects

The biological responses to glucocorticoids may differ between or within animal species. For example, lymphocytes from rodents are lysed by hydrocortisone, while hydrocortisone reduces the number of human lymphocytes by inducing their migration from circulation. Human and mouse cells respond differently to glucocorticoids in culture (Runikis et al., 1978). Proliferation of L-929 cells during log growth is inhibited by glucocorticoids (Pratt and Aronow, 1966; Berliner and Ruhmann, 1967) but human skin fibroblasts have increased growth rate under the same conditions (Runikis et al., 1978; Kirk and Mittwoch, 1977). Moreover, the effect of glucocorticoid on collagen synthesis is often reported as inhibitory, yet the stimulation effect has also been claimed (Harvey et al., 1974; Doherty and Saarni, 1976). These discrepant observations have no reasonable explanation, but underline the importance of reporting cell types used for experiments.

In addition, even within the same cell line, dichotomic effect is observed. Stormshak reported that chronic injections of estrogens into ovariectomized mice initially stimulated, then depressed uterine cell division, DNA synthesis, and DNA polymerase activity. It clearly demonstrated a dose- and time-dependent refractoriness (Stormshak et al., 1978). A time-dependence of steroid action also is demonstrated in another similar study (Glasser et al., 1972). Injection of estradiol

into the immature rat stimulated an increase in uterine DNA synthesis by 18 hr after treatment. Maximal synthesis of DNA occurred by 24 hr then subsided to control level by 36 hr. The effect of estradiol on DNA polymerase activities followed a similar course.

Therefore, it is of particular interest to elucidate the time profile of glucocorticoid-induced biochemical events which occur during prolonged periods of exposure to glucocorticoid. The time-dependence of steroid action may be another important factor other than cell-type and concentration-dependences in rationalizing the dichotomy of glucocorticoid effects.

A recent study of the effect of time and concentration on the action of hydrocortisone on fibroblasts in vitro measuring DNA, hyaluronic acid and protein synthesis (Saarni and Tammi, 1978) demonstrated several impressive effects in DNA synthesis. They observed a lower ^{3}H-thymidine uptake by hydrocortisone-exposed cells than by control cells, demonstrating that the hydrocortisone inhibits scheduled DNA synthesis (at the level of 10^{-5} M). Similarly, they found that both glucocorticoid stimulation and inhibition of the formation of several metabolites on the pathway of the connective tissue synthesis depended on the glucocorticoid incubation time.

This discovery, together with the uncertain mechanism of steroid action, leads to the conclusion that studies of the secondary effects of glucocorticoid, such as collagen metabolism, RNA synthesis, cell proliferation, either in cultured cells or in the skin, have only a limited value until the more fundamental questions of glucocorticoid primary effect(s) on nuclear acceptor sites have been clarified.

XV. Subcellular localization of glucocorticoids

Studies of subcellular localization of glucocorticoids have been

relatively limited. None have been done with skin cells other than mouse L-929 fibroblasts. Most of the work done has dealt only with the distribution between cytoplasm and nucleus. The techniques employed in these studies generally have involved the lysis of whole cells followed by ultracentrifugation of the crude lysate. This approach provides less precise information concerning the glucocorticoid localization between cytoplasm and nuclei than isolation of intact nuclei prior to the measurements of the retained radioactive glucocorticoids.

In respect to studies of the distribution of glucocorticoids within the nuclear compartment, there have been even fewer studies reported. Middlebrook et al. (1975) distinguished two types of nuclear retention of glucocorticoids, designated as nuclear extractable and nuclear residual portions, based on differential extractability of these two fractions by 0.3 M KCl. These investigations did not correlate clearly with any specific component of the nucleus. Higgins et al. (1979) reported in their recent review article that glucocorticoid-receptor complexes have been found in chromatin but not in the nucleolus, nucleoplasma or nuclear membrane. Very little is known about the glucocorticoid distribution within a nucleus.

The subchromatin localization of glucocorticoids within the chromatin is equally poorly understood. Retention of glucocorticoid in chromatin has been associated with the internucleosomal regions or with the DNAse-sensitive portion of the nucleosome, since DNAse I, which preferentially attacks the internucleosomal regions of chromatin, abolishes the retention of glucocorticoid in chromatin (Baxter et al., 1972; Higgins et al. 1973b; Noll, 1976). Bloom and Anderson (1978) have used the hydroxyapatite dissociation method to fractionate the chromatin into unbound chromosomal proteins, loosely-bound non-histone protein, histone

proteins (H_1, H_2A & H_2B, and H_3 & H_4), and tightly bound non-histone protein. They found the estrogen-receptor complexes approximately 70% eluted with loosely bound non-histone protein, less than 5% with histone proteins, and 25% with tightly bound non-histone proteins. No such a study was known for glucocorticoids, and no precise subchromatin localization of glucocorticoid has been confirmed.

The retention of glucocorticoids in nuclei or chromatin as a function of time has been generally studied only up to 6 hr. It is of particular interest to examine their behaviour for longer periods, since chromatin is not a static entity, but undergoes many chemical and structural changes (Pederson, 1972). Such changes might well affect the amount of glucocorticoid retained as well as the type of interaction between chromatin and complex. Anderson et al. (1973) studied the correlation of estrogen-induced uterotropic and growth responses with nuclear steroid-receptor complex content. They found that, in general, growth responses were proportional to the quantity of estrogen-receptor complex that remained bound to the nucleus for 6 hours, not the quantity driven into the nucleus immediately after administration of large dose of estradiol in vivo. Thus, nuclear retention, rather than initial nuclear uptake, of receptors, determined such complex responses as cellular growth and replication (O'Malley and Buller, 1976).

The time profiles of glucocorticoid retention in various subcellular fractions, are reported in this thesis to investigate the time dependence of glucocorticoid subcellular localization.

EXPERIMENTAL

The "Outline of Research" shows the inter-relationship of the various experiments and methods comprising this thesis as related to the three types of experiments:

(1) Gas-liquid chromatographic assay of hydrocortisone, triamcinolone acetonide and desonide.

(2) Isolation of DNA from intact cells and examination of these DNA for cross-linking with non-radioactive glucocorticoids over incubation periods of varying lengths of time up to 96 hours.

(3) Examination of subcellular distribution of tritiated TA under intact cell conditions for determining:

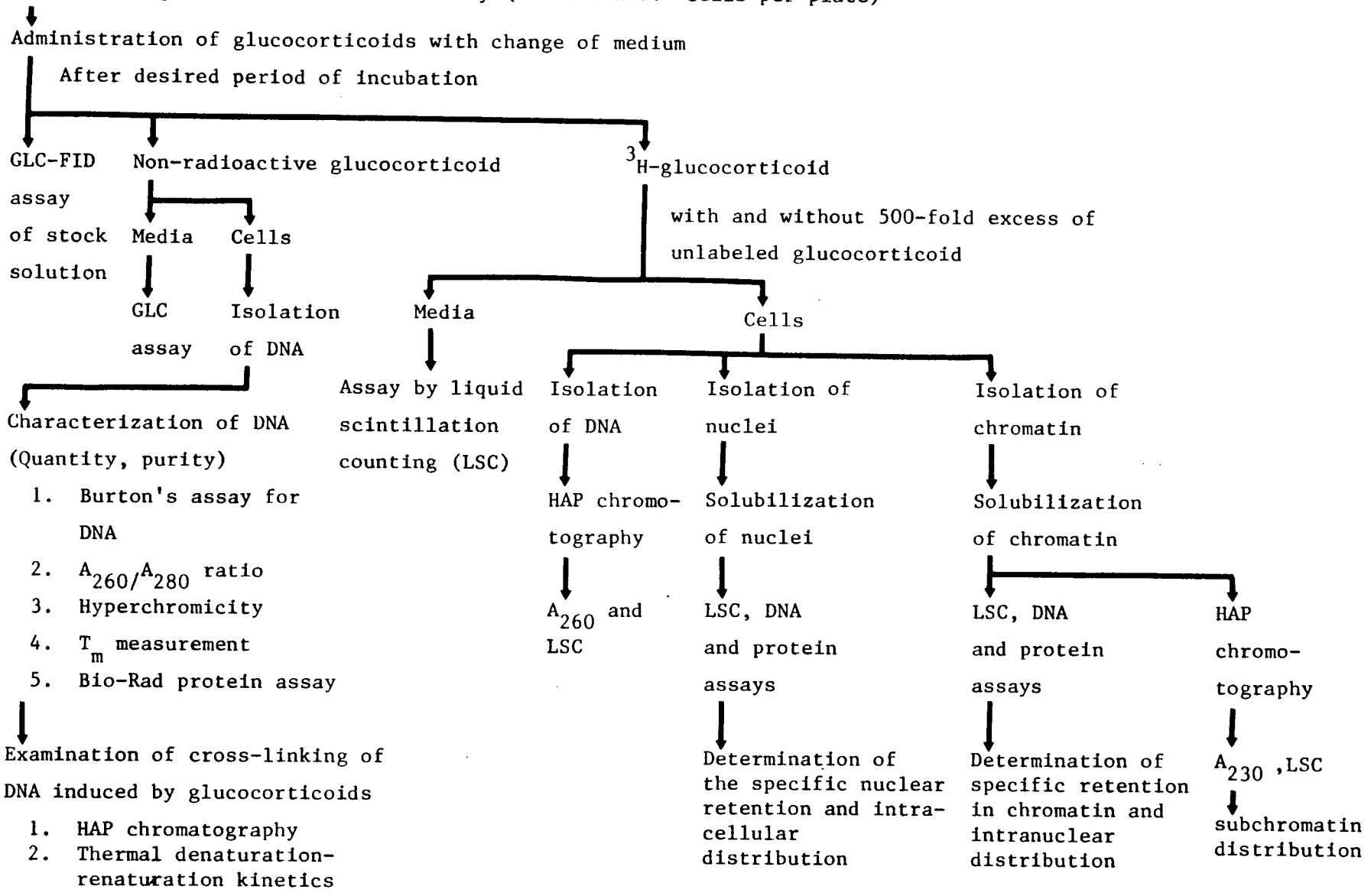
(a) retention of ^{3}H-TA in DNA,

(b) retention of ^{3}H-TA in nuclei and intracellular distribution of ^{3}H-TA,

(c) retention of ^{3}H-TA in chromatin and intranuclear distribution of ^{3}H-TA,

(d) subchromatin distribution of ^{3}H-TA.

Outline of the Research



Section I. Gas-liquid chromatographic analysis of hydrocortisone, triamcinolone acetonide and desonide in culture media of mouse and human dermal fibroblasts

A quantitative gas-liquid chromatographic (GLC) assay with flame-ionization detection (FID) was developed. The development and the applicability of this assay are described in this section.

(A) Introduction

Synthetic glucocorticoids vary in their clinical potencies as well as in their potential for causing dermal atrophy, the major adverse effect of long-term topical glucocorticoid therapy (Stewart et al., 1978). The majority of the studies on the pathogenesis of this adverse effect have used cultured dermal cells as models, and the incubation of glucocorticoids with cultured cells has lasted from hours to days. However, no assays of glucocorticoids in culture media appear to have been published. It has been assumed that the amounts of glucocorticoids added to cell culture media represent their biologically active concentrations and the metabolism of glucocorticoids during the period of incubation has been negligible. The development of an assay, therefore is an important test of the validity of such assumptions.

The requirements for quantitative assay in biological systems are that sensitivity should be in the low nanogram range because glucocorticoids are physiologically active at these low concentrations, and the technique should be able to identify possible metabolites or decomposition products. Due to ready instrument availability, GLC-FID assay was investigated for the determination of glucocorticoids in cell culture systems.

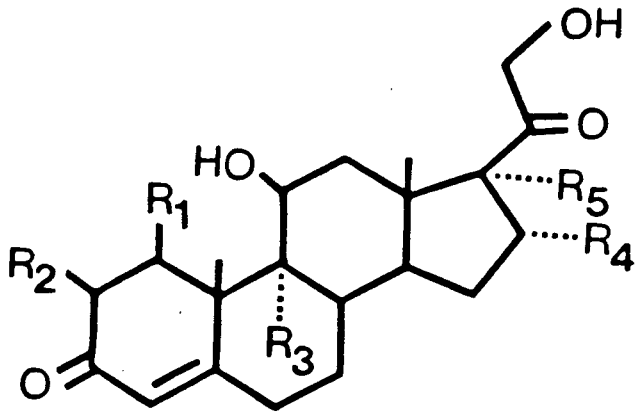
Few quantitative HPLC (Ryrfeldt et al., 1979; Loo et al., 1977; Loo and Jordan, 1977; Wortman et al., 1973; Roth et al., 1980) and GLC (Martin and Amos, 1978; Mason and Fraser, 1975; Simpson, 1973) assays have been reported for the analysis of synthetic glucocorticoids in biological fluids. Among HPLC assays, the work of Ryrfeldt et al. (1979) measuring plasma levels of budesonide in dogs with the aid of radioactive detection achieved sensitivity as low as 0.05 ng/mL plasma. Among GLC techniques, Simpson (1973) estimated triamcinolone acetonide, triamcinolone and prednisolone in rat muscle, using electron capture detection and trimethylsilyl derivative,with sensitivities between 2 to 4 ng of the glucocorticoids. Martin and Amos (1978) reported a GC-MS assay for prednisone and prednisolone as methoxime-trimethylsilyl derivative to ng/mL plasma levels. Methoxime-trimethylsilyl derivative had not been applied to the assay of other synthetic glucocorticoids (Martin and Amos, 1978), although it had been used successfully for the assays of naturally-occurring steroids (Prost and Maume, 1974). No specific assay techniques have been reported so far for desonide.

(B) Experimental

(1) Materials

The descriptions of chemicals, reagents and apparatus used are compiled in Appendices I and II.

The test glucocorticoids included the synthetic fluorinated glucocorticoids, triamcinolone acetonide (TA) and triamcinolone (T), their non-fluorinated analog, desonide (DSN) and the naturally occurring hydrocortisone (HC). Their chemical structures are shown in Figures 19. Methanol and ethyl acetate were distilled in glass, and pyridine was the silylating grade.



GLUCOCORTICOID	R_1	R_2	R_3	R_4	R_5
Hydrocortisone	H	H	H	H	OH
Triamcinolone	1 Δ		F	OH	OH
Triamcinolone acetonide	1 Δ		F	O⟍⟋O	
Desonide	1 Δ		H	O⟍⟋O	

Figure 19 : Chemical structures of test glucocorticoids

Methoxyamine hydrochloride, dissolved in pyridine, 100 mg/mL (Pfaffenberger and Horning, 1975), was stored in a conical Reacti-vial® fitted with a Teflon-lined Mininert valve® at 4°C in a desiccator.

N-trimethylsilyl imidazole (TMSI), was transferred to a conical Reacti-vial® fitted with a Mininert valve® immediately after each ampoule was opened, and the vial was kept at 4°C in a desiccator.

(2) Preparation of derivatives

Methoxime-trimethylsilyl (MO-TMS) derivatives: The procedure of Pfaffenberger and Horning (1975) was adapted as follows:

Test glucocorticoid (not more than 800 µg) was placed in 1 mL Reacti-vial® fitted with a Teflon-lined screw cap. One hundred µL of methoxyamine hydrochloride/pyridine stock solution was added to dissolve the compound, and the resulting solution was heated at 70°C for 15 minutes. One hundred µL of TMSI was added and silylation was conducted at 100°C for 10 to 30 minutes depending on the compound tested. One to 2 µL of the final reaction mixture (volume of 200 µL) was injected directly into the GLC or GC-MS.

t-Butyldimethylsilyl (BDMS) derivatives: The procedure was modified from those reported in the literature (Gaskell and Brooks, 1976; Kelly and Taylor, 1976; Phillipou et al., 1975). Test glucocorticoid (not more than 800 µg) was placed in a 1 mL Reacti-vial® fitted with Teflon-lined screw cap. One hundred µL of t-butyldimethylsilyl chloride mixture (1.0 mmole t-butyldimethylsilyl chloride, 2.5 mmole imidazole per mL of anhydrous N,N-dimethylformamide) was added to dissolve the compound, and the resulting solution was incubated at 100°C for 1 hour. After the reaction, 100 µL of distilled water was added and the derivatives were extracted into chloroform prior to GLC or GC-MS analysis.

(3) Differential scanning calorimetry

A differential scanning calorimeter was employed. All samples were crimped. The rate of temperature increase was 10°C/min for each run.

(4) Gas-liquid chromatography

A Hewlett-Packard gas-liquid chromatograph (Model 5830A) equipped with a terminal and a flame-ionization detector was used. The coiled glass column, 1.8 m x 2 mm i.d., contained 3% OV-7 coated onto 80-100 mesh Chromosorb W (HP).

All columns were conditioned at 100°C for 15 hours, and then the oven temperature was increased by 0.1°C/min up to 285°C, at which it was allowed to remain for approximately 15 hours. The flow rate of carrier gas during the conditioning period was 10 mL/min.

The operating temperatures for routine analysis were: injection port, 265°C; column, 285°C and detector, 300°C. The gas flow rates were: carrier gas (helium), 60 mL/min; hydrogen, 40 mL/min; and air, 300 mL/min.

(5) Gas chromatography-mass spectrometry

A computerized Varian MATT-111 gas chromatograph-electron-impact mass spectrometer was used to study the fragmentation pattern of the MO-TMS derivatives for confirming the chemical structures of the derivatives. The following conditions were used for the GC: injection port temperature, 230°C; column temperature was programmed from 200°C to 260°C at a rate of 10°C/min; and carrier gas (helium) flow rate, 20 mL/min. The 2 m x 2 mm i.d. glass column was packed with 3% OV-17 coated onto 80-100 mesh Chromosorb W (HP). For the MS, the ionization beam energy was 80 eV; the source analyzer temperature was 250°C: and the separator was at 280°C.

The modes of detection included total ion current and selected ion monitoring.

(6) Extraction procedure

The glucocorticoids were double extracted from the cell culture medium containing 10% fetal calf serum (FCS) in Dulbecco Modified Eagle Medium (DMEM) as follows:

Solid sodium chloride was added to 5 to 10 mL aliquots of the medium, decanted from cell cultures, to make it 1 N. The aqueous phase was extracted twice with an equal volume of glass-distilled ethyl acetate. After centrifugation to separate the layers, the organic extracts were combined and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residue was transferred to a 1 mL Reactivial® containing 100 µL of internal standard (0.50 µg/mL in methanol) by rinsing three times with 0.2 mL of fresh ethyl acetate. The resulting mixture was evaporated to dryness and subjected to derivatization.

A white interfacial precipitate occurred in the extraction of glucocorticoids from serum-containing medium. The addition of NaCl to make a 1 N solution avoided this difficulty.

(7) Recovery studies

A series of known amounts of test glucocorticoid solution in methanol (HC, TA or DSN) were added to individual centrifuge tubes and evaporated to dryness under a stream of nitrogen. Five mL of 10% FCS-containing medium, either freshly prepared or decanted from control cells were added to each glucocorticoid residue. The spiked samples were extracted by the procedure described above. Percentage recoveries were calculated from standard curves obtained from glucocorticoid samples which were derivatized without extraction.

(8) Assays on biological samples

The glucocorticoid stock solution as well as the glucocorticoid-containing media were assayed before addition to the cells to ascertain the extent of agreement between calculated and observed glucocorticoid concentrations. Deviations of more than 15% of expected values were observed on occasion because of dilution errors. Only experimentally determined concentrations have been reported in the following tables.

The glucocorticoid-containing medium was incubated with either mouse L-929 or human dermal fibroblasts after the cells had reached confluence in duplicate culture plates for time periods up to 96 hours. The decanted media from the 2 plates containing 6 to 8 x 10^6 cells were pooled and stored at -4°C until analysis. The cells were simultaneously harvested for biochemical studies of glucocorticoid effects on cellular DNA reported later in this thesis.

(C) Results and discussion

(1) Confirmation of purities of test glucocorticoids

All steroids were dried at 40°C for 1 hour, and at ambient temperature overnight in a vacuum oven before use. The weight lost due to the drying was less than 1% in all cases.

The melting points of these compounds were determined by both capillary m.p. determination and differential scanning calorimetry. The purities of HC, DSN and PRG as received were satisfactory. The samples of T and TA had to be recrystallized by dissolving the compound in methanol with the aid of steam, and allowing the filtered resulting solution to stand in a hood for three days before collecting the crystals by filtration. The purities of all compounds were confirmed by HPLC.

(2) Selection of extracting solvent

Solvents of ethyl acetate, diethyl ether, chloroform and methylene chloride were evaluated for their extraction efficiency of glucocorticoid from serum-free and serum-containing media. Ethyl acetate was chosen, because it had higher extraction efficiency, and permitted easier withdrawal of extracts.

The presence of serum in the medium decreases the extractability of glucocorticoid by single extraction and, therefore, double extraction was used as a routine procedure.

(3) Selection of derivatizing agents

For initial experiments, t-butyldimethylsilyl chloride was selected as a potential derivatizing reagent based on the advantages described in the literature (Gaskell and Brooks, 1976; Kelly and Taylor, 1976; Phillipou, 1975). These were (i) high selectivity of the reaction by which silylation occurs only at the unhindered C_{21}-OH group of the glucocorticoid, (ii) increased stability of t-butyldimethylsilyl ethers towards hydrolysis, which is 10^4 times higher than that of trimethylsilyl ethers, and (iii) the simplicity of the mass spectra of t-butyldimethylsilyl ethers, because these compounds give intense $(M-57)^+$ ions. By monitoring this characteristic ion, MS has the potential to be a sensitive and selective technique for quantitative analysis of glucocorticoids. The results obtained with this derivative, however, were generally disappointing. The t-BDMS derivatives had unsatisfactory GLC properties, such as retention times longer than those of their parent compounds and adsorption to the column occurred due to the remaining free functional groups. Therefore, the further use of t-butyldimethylsilyl chloride was discontinued.

The MO-TMS derivatives of test glucocorticoids proved to be superior to their corresponding t-BDMS derivatives based on their short retention times and sharp symmetrical peaks with higher molar response than their parent compounds.

(4) Column selection

Columns packed with 3% OV-7, OV-17 and OV-25 were examined for their suitability to resolve peaks of the internal standard, glucocorticoids and any endogenous component in serum. The OV-7 and OV-17 columns gave superior elution profiles to the OV-25 column. However, with OV-17, the serum associated peaks prevented satisfactory resolution of the internal standard, PRG, a difficulty not present with the OV-7 column (Figure 20-b and 20-c). Therefore, OV-7 packing was selected for the assay notwithstanding the longer retention times observed (Table VI).

(5) Optimum reaction time for silylation and stability of derivatives

The optimum time for silylation was evaluated by reacting samples containing equivalent amounts of HC, T or TA for various times at 100°C. Known amounts of separately derivatized methoxime internal standards were then added. The yield of the derivative, as observed by its peak area ratio to that of the internal standard, was monitored. Figure 21 showed that the optimum yield was obtained when reaction times were 10, 15 and 30 minutes for HC, T and TA respectively.

The MO-TMS derivatives were stable in excess of 24 hours at 4°C when stored in tightly capped reaction vials.

(6) Confirmation of derivative formation using GC-MS

The fragmentation patterns of derivatized HC, T, TA and DSN when analyzed by electron impact GLC-mass spectrometry confirmed the formation of MO-TMS derivatives and PRG as the MO derivative (Figure 22 a-e). There is little information available on the fragmentation of the MO-TMS

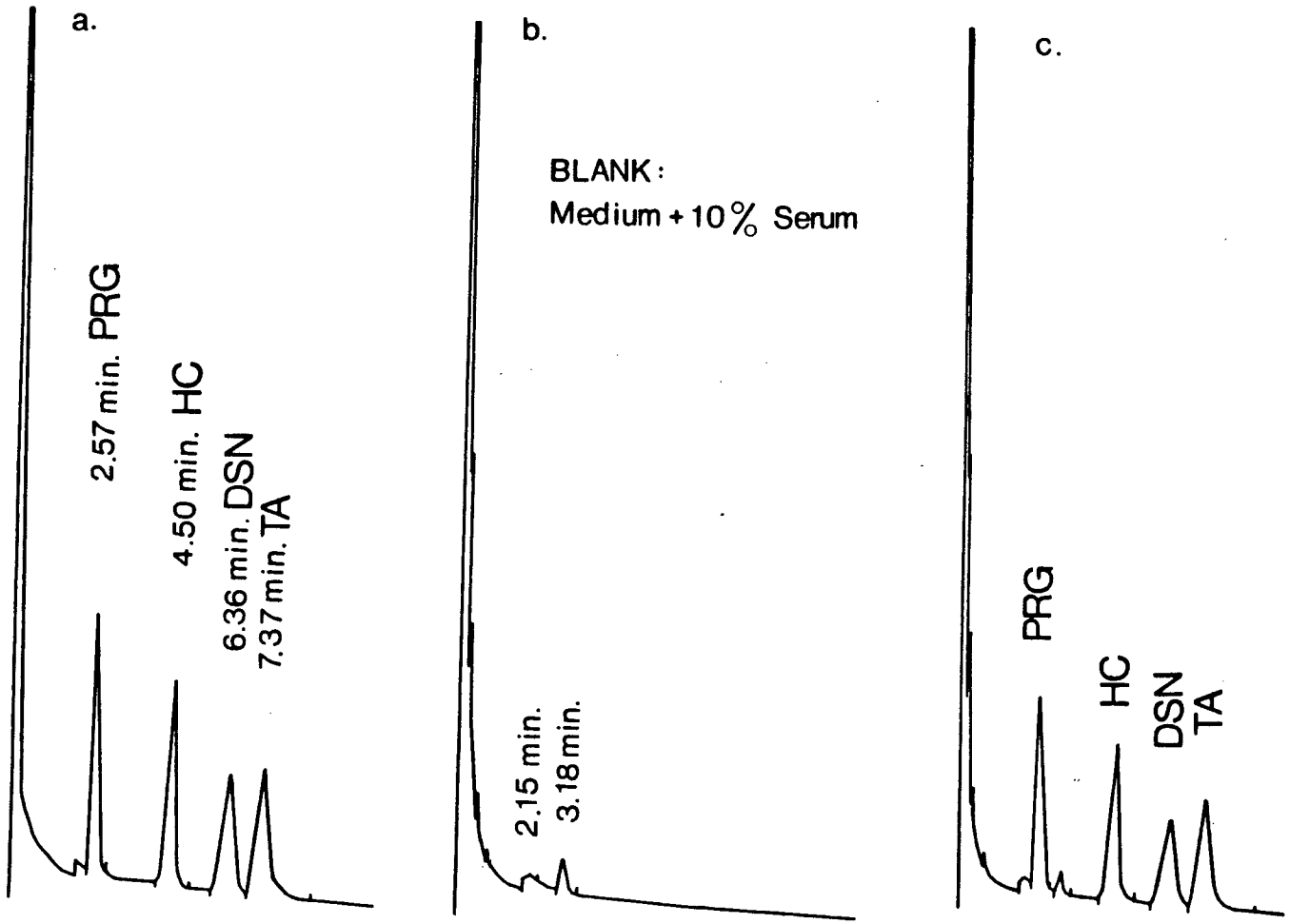


Figure 20 : Typical chromatograms of glucocorticoids and extracts

(a) Authentic glucocorticoids (b) Extract of blank medium with 10% serum

(c) Glucocorticoids extracted from serum-containing medium

Table VI Retention time of parent and derivatized glucocorticoids

Glucocorticoid	Retention time, min[a]		
	Underivatized compound[b]	MO-TMS derivative	
	3% OV-17	3% OV-17	3% OV-7
Progesterone	1.31	1.38	2.57
Prednisolone	-	2.20	-
Hydrocortisone	4.09	2.10	4.50
Triamcinolone	5.04	3.53	-
Desonide	7.25	3.69	6.36
Triamcinolone acetonide	8.59	4.38	7.37

(a) Three or more values are averaged.

(b) All compounds except progesterone gave more than one peak due to thermal decomposition. The value given is that of the major peak of each sample.

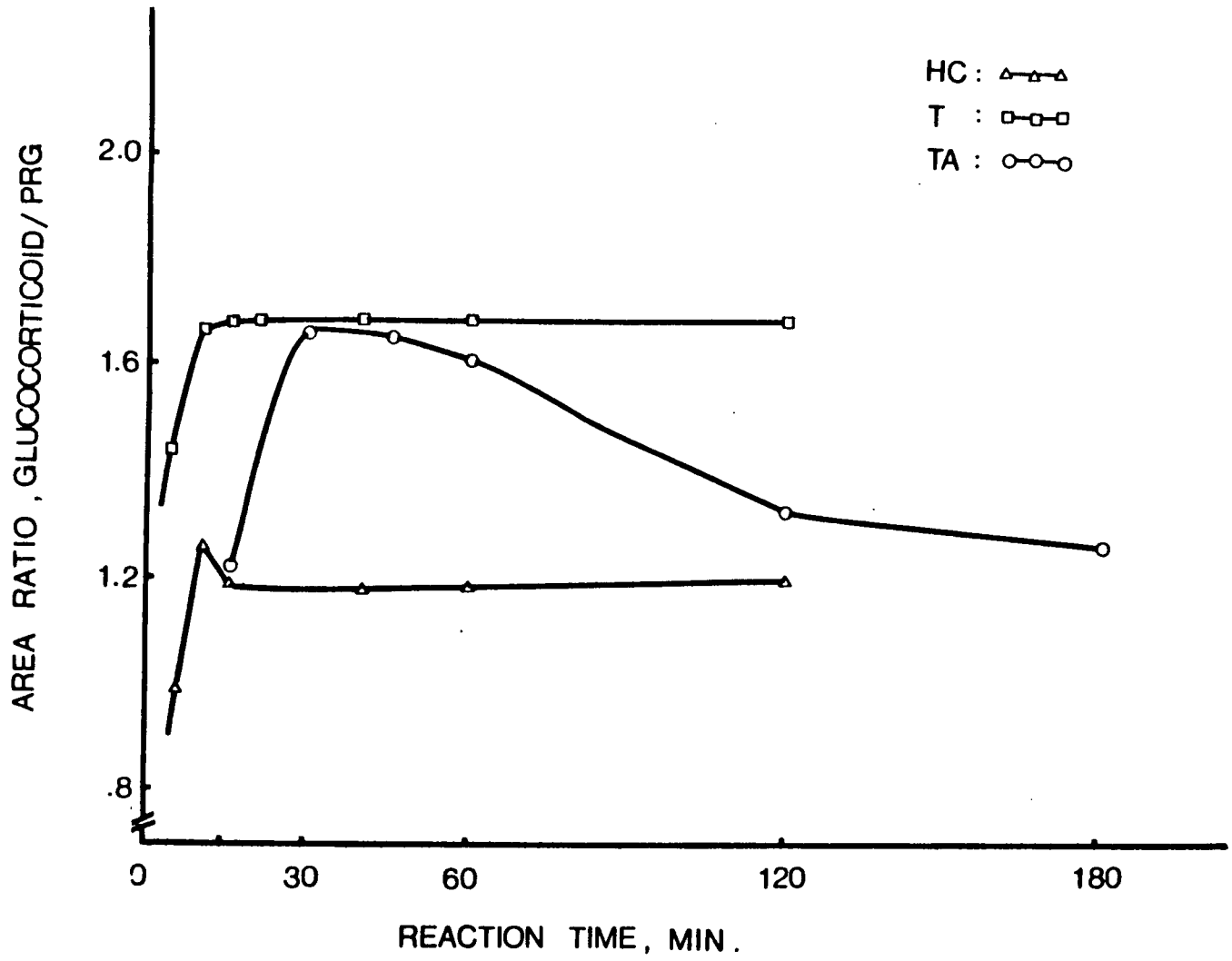


Figure 21 : Reaction kinetics of HC, T and TA with silylating agent at 100°C

Plot of area ratio of derivatized compounds to derivatized PRG as a function of time

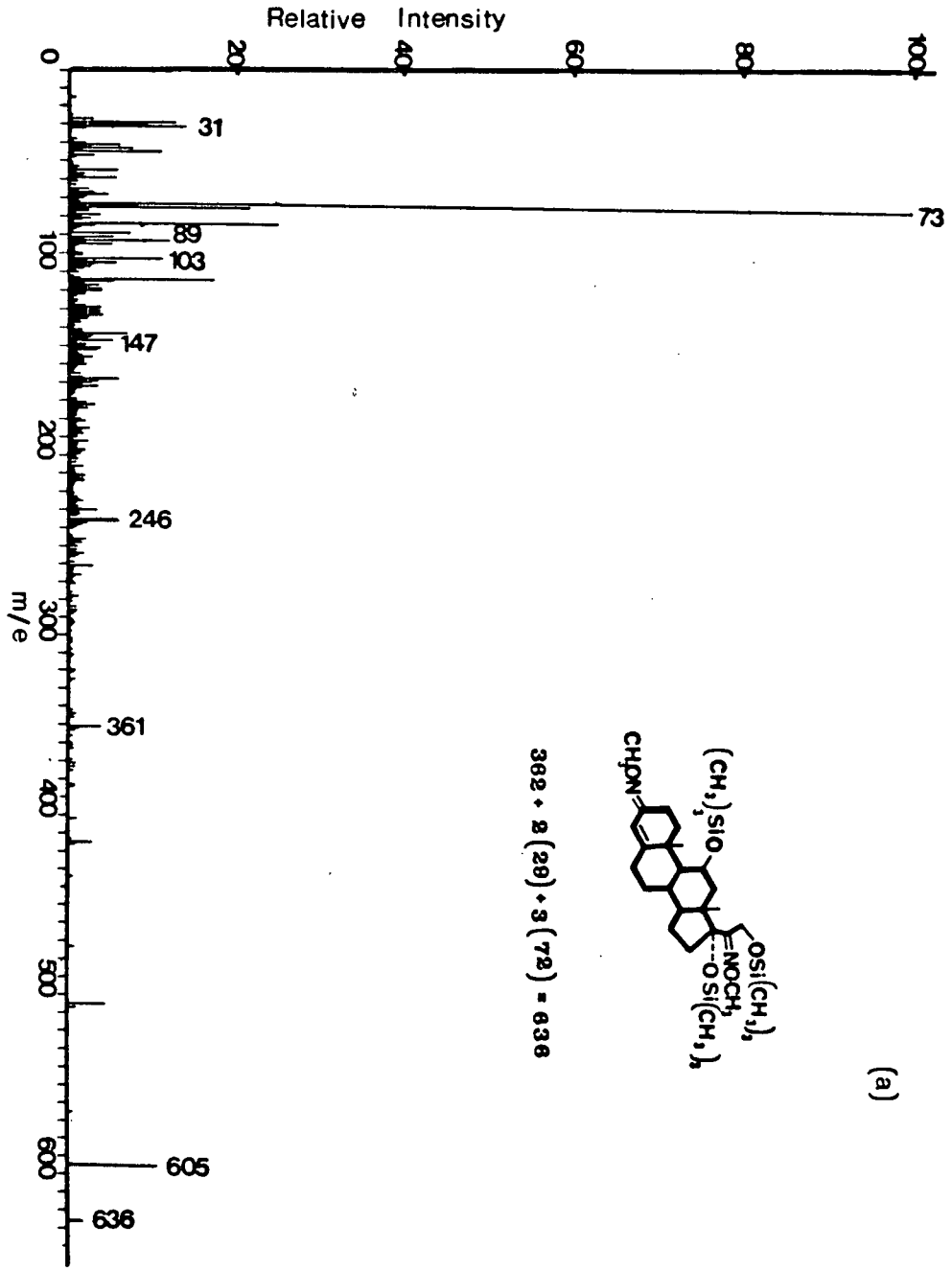


Figure 22-a : Electron-impact mass spectrum of $(MO)_2$-$(TMS)_3$-HC

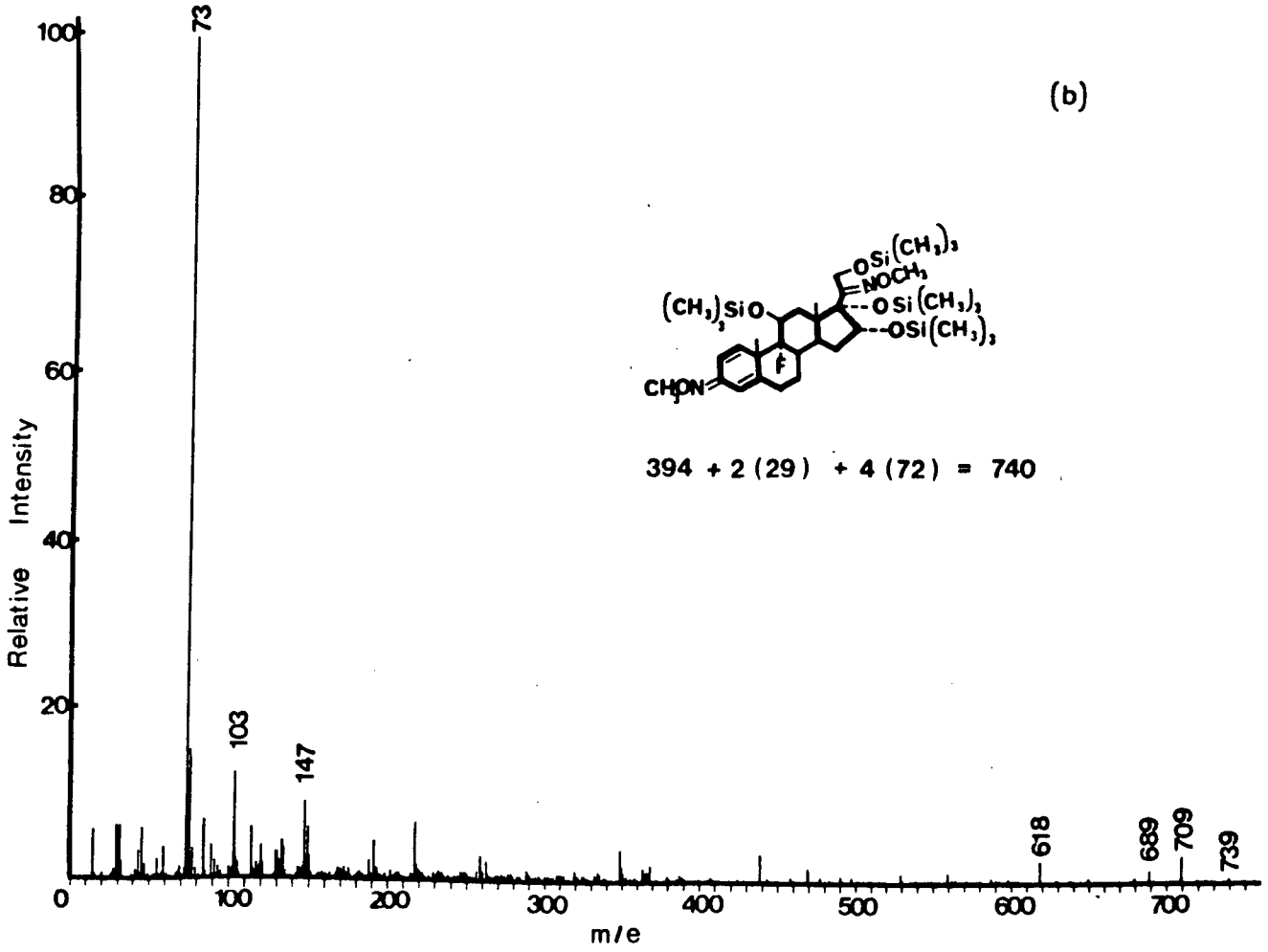


Figure 22-b : Electron-impact mass spectrum $(MO)_2$-$(TMS)_4$-T

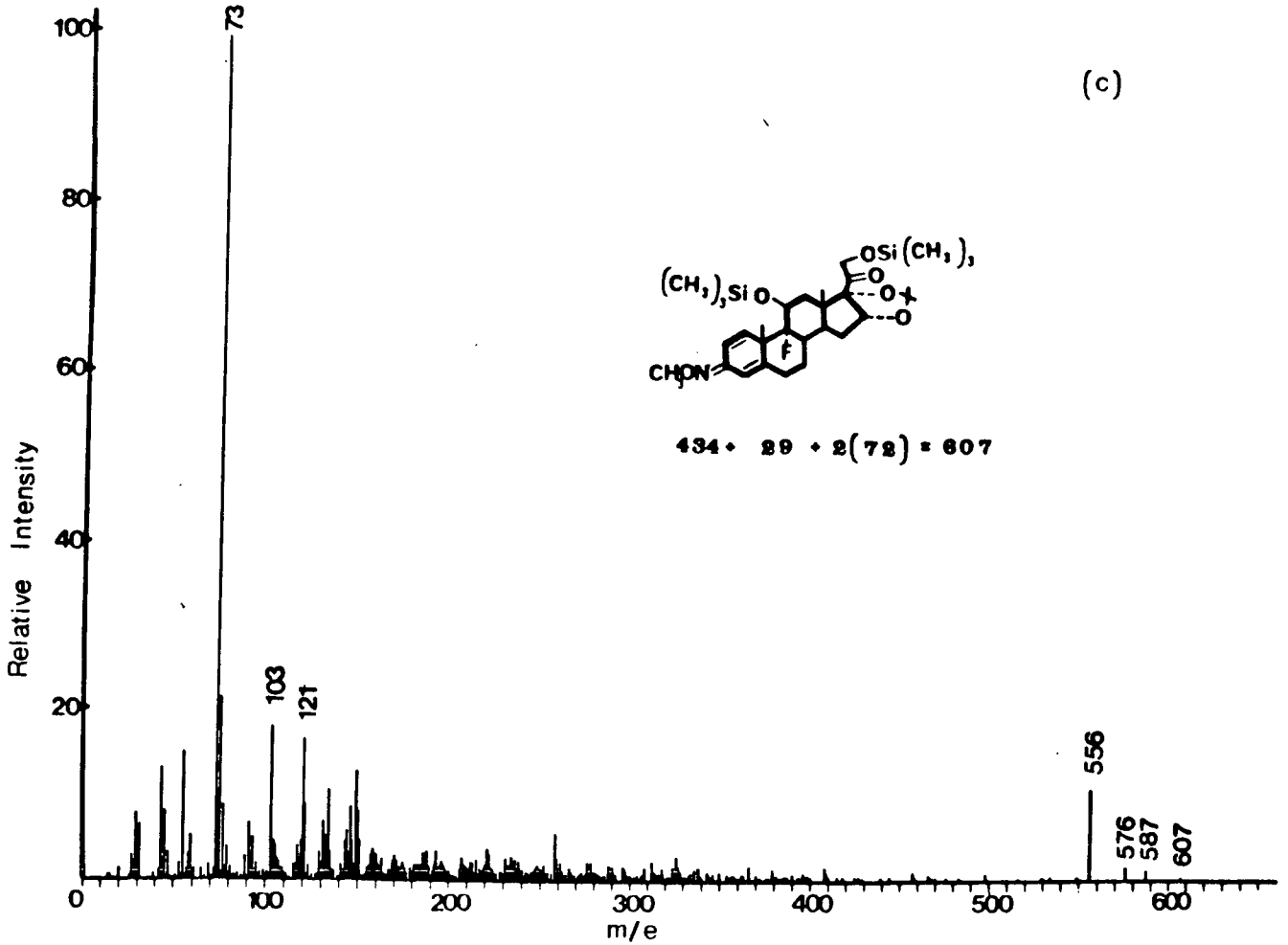


Figure 22-c : Electron-impact mass spectrum of $(MO)_1$-$(TMS)_2$ - TA

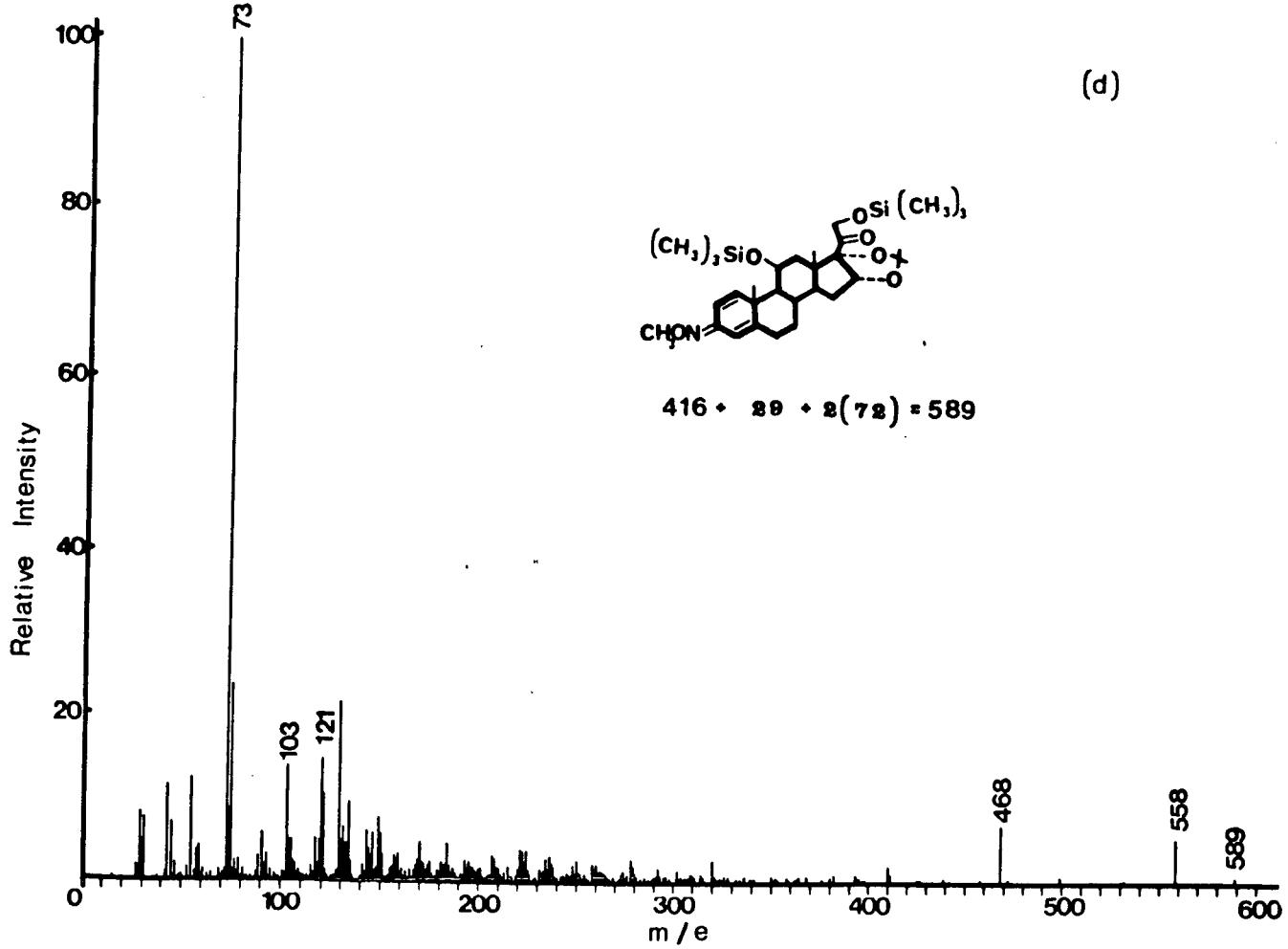


Figure 22-d : Electron-impact mass spectrum of $(MO)_1$-$(TMS)_2$- DSN

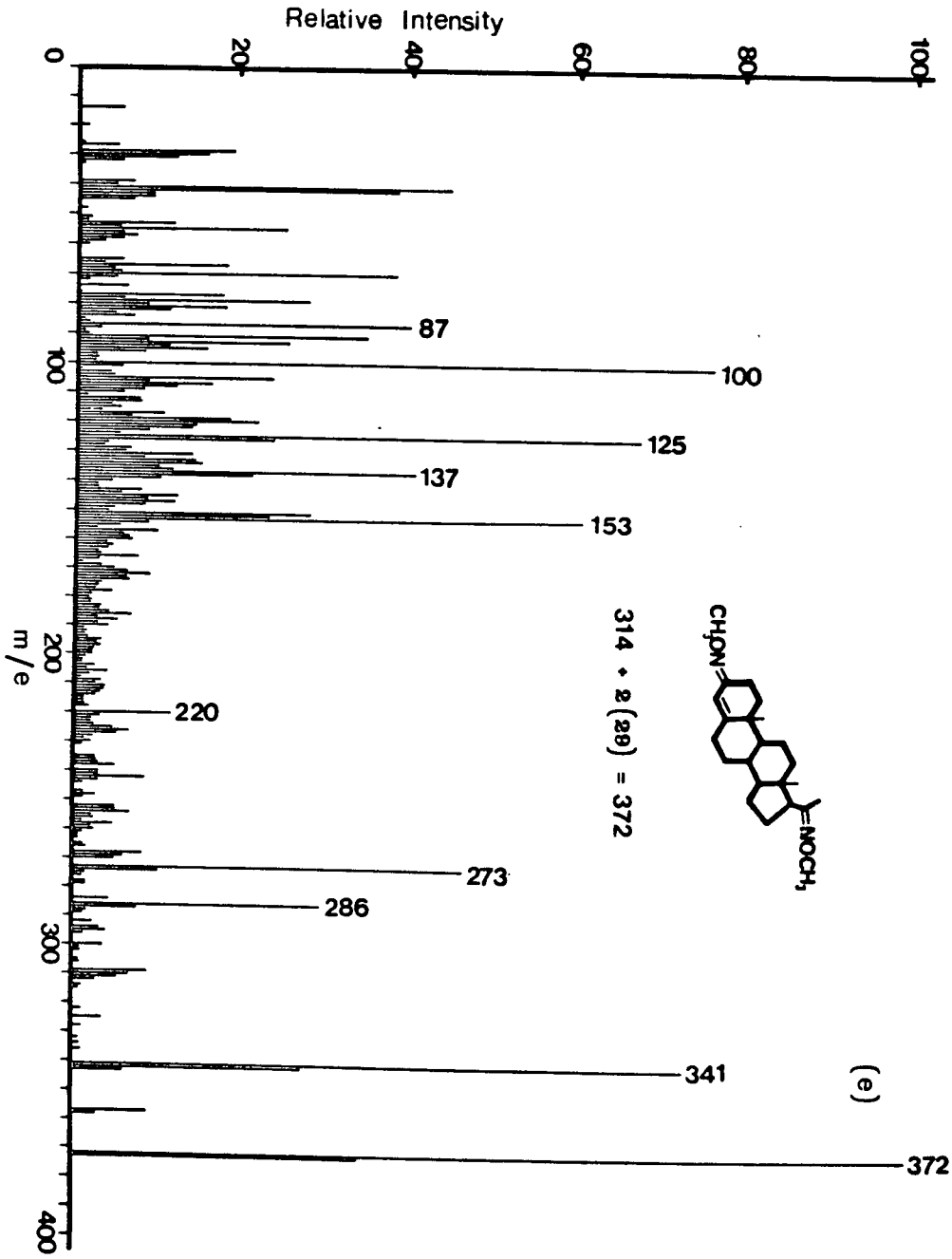
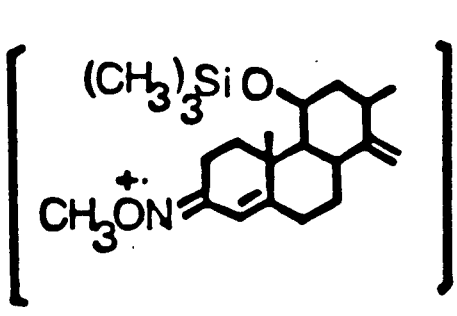


Figure 22-e : Electron-impact mass spectrum of $(MO)_2$ - PRG

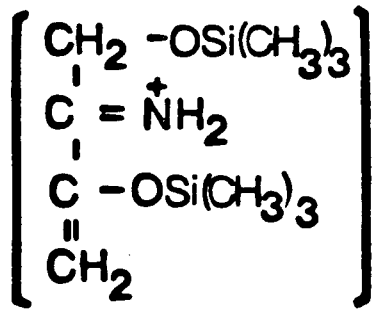
derivatives of the synthetic glucocorticoids. Those of steroids present in adrenal gland extracts of rats, primarily corticosterone derivatives, have been reported by Prost and Maume (1974). Their scheme of characteristic cleavage form the basis for the interpretation of the observed spectra (Figure 22 a-e). The well known losses of O-methoxy (m/e 31) from O-methoxime groups and of silanol (m/e 89) from trimethylsilyl groups (Prost and Maume, 1974; Harvey and Vouros, 1979) were observed in these reported experiments.

(a) Fragmentation pattern of HC derivative, $(MO)_2$-$(TMS)_3$-HC

The peak at m/e 636 (1.8%) corresponds to the molecular ion peak; other characteristic peaks were observed at m/e 605 (10.5%) $\left((M-OCH_3)^+\right)$, 361 (3.5%), 246 (6.1%), 147 (7.0%) $\left((CH_3)_2Si = \overset{+}{O}Si(CH_3)_3\right)$ (Engel and Orr, 1972), 103 (11.4%) $\left(CH_2 = \overset{+}{O} - Si(CH_3)_3\right)$, 89 (12.3%) $\left(\overset{+}{O} - Si(CH_3)_3\right)$, and 73 (100%) $\left(\overset{+}{Si}(CH_3)_3\right)$.



m/e 361



m/e 246

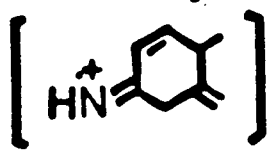
(b) Fragmentation pattern of T derivative, $(MO)_2$ - $(TMS)_4$ - T

The peak at m/e 739 (0.7%) corresponds to one less thn the molecular ion $\left((M-1)^+\right)$. The characteristic peaks are 709 (3.0%) $\left((M-OCH_3)^+\right)$, 689 (1.4%) $\left((M-OCH_3-HF)^+\right)$, 618 (2.6%) $\left(M-F-(CH_2 = OSi(CH_3)_3)\right)^+$, 147 (8.8%), 103 (12.3%) and 73 (100%).

(c) Fragmentation pattern of TA derivative, $(MO)_1$ - $(TMS)_2$ - TA

The peak at m/e 607 (0.4%) corresponds to the molecular ion.

The characteristic peaks are 587 (1.1%) $\left((M-HF)^{+\cdot}\right)$, 576 (1.6%) $\left((M-OCH_3)^{+}\right)$, 556 (10.7%) $\left((M-OCH_3-HF)^{+\cdot}\right)$, 121 (9.0%), 103 (18.1%) and 73 (100%).



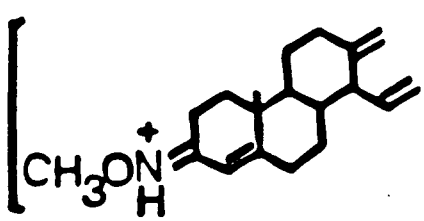
m/e 121

(d) Fragmentation pattern of DSN derivative, $(MO)_1$ - $(TMS)_2$ - DSN

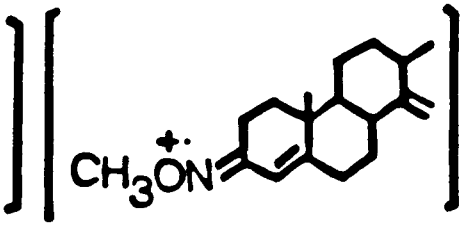
The peak at m/e 589 (0.8%) corresponds to the molecular ion. The characteristic peaks are 558 (4.9%) $\left((M-OCH_3)^{+}\right)$, 468 (6.8%) $\left((M-121)^{+\cdot}\right)$, 121 (10.1%), 103 (13.7%) and 73 (100%).

(e) Fragmentation pattern of PRG derivative, $(MO)_2$ - PRG

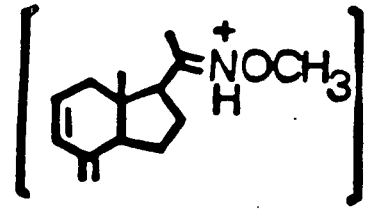
The peak at m/e 372 (100%) corresponds to the molecular ion. The characteristic peaks are 341 (72.8%) $\left((M-OCH_3)^{+}\right)$, 286 (29.5%), 273 (46.2%), 220 (11.5%), 153 (57.4%), 137 (40.2%), 125 (65.6%), 100 (75.5%) and 87 (39.1%).



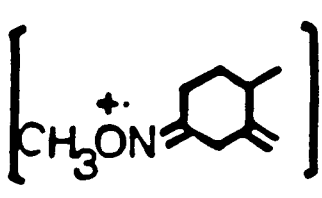
m/e 286



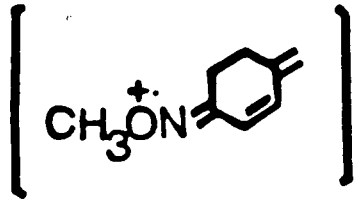
m/e 273



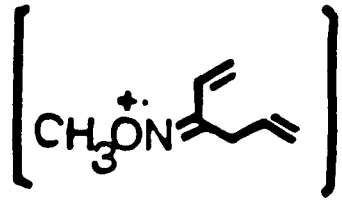
m/e 220



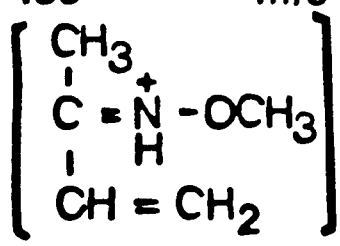
m/e 153



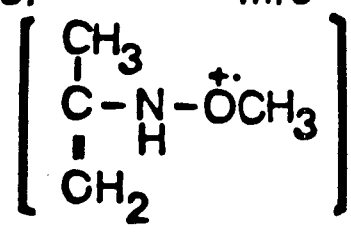
m/e 137



m/e 125



m/e 100



m/e 87

(7) Resolution of MO-TMS derivatives on OV-7 and OV-17 columns

The GLC chromatograms of all four glucocorticoid derivatives appeared as single peaks when eluted from OV-7 columns (Figure 20-a and 20-c). Compounds TA and DSN, however, resolved into two peaks, a minor peak A with shorter retention time and occupying less than 10% of peak area, and a major one B, when OV-17 packings were used due to a higher degree of resolution obtainable with OV-17 columns.

A GC-MS examination showed that the chemical structure of the derivative eluting as the minor peak A corresponded to $(MO)_1$ - $(TMS)_3$ - DSN, m/e = 660 $(M-1)^+$ whereas that of the major peak B corresponded to $(MO)_1$ - $(TMS)_2$ - DSN, m/e = 589 (m^+) as illustrated in Figure 23-a and 22-d, respectively.

We ascribe the formation of peak A as being due to partial enolization of the keto group at C_{20}. The keto group at C_{20} in DSN as well as in TA, in contrast to HC and T, is hindered by the C_{16}, C_{17} acetonide group and remains largely resistant to formation of the MO derivative. Under favourable conditions such as exposure of the reaction mixtures to sunlight radiation, and excess silylating reagent, some enolization, however, can occur which then would lead to the formation of the minor $(TMS)_3$-derivative observed.

Indirect evidence for the existence of the enolization reaction was obtained upon exposure of the reaction mixture to sunlight. Sunlight has been long recognized as a catalyst for the enolization of a wide variety of ketones (Hart, 1979). Exposure to sunlight increased the relative magnitude of peak A to peak B as expected of a sunlight-catalyzed reaction. Removal of the reaction mixture from sunlight reversed the magnitude of peak A back to its original levels, indicating the reversibility of the sunlight-induced enolization reaction. Prolonging deriva-

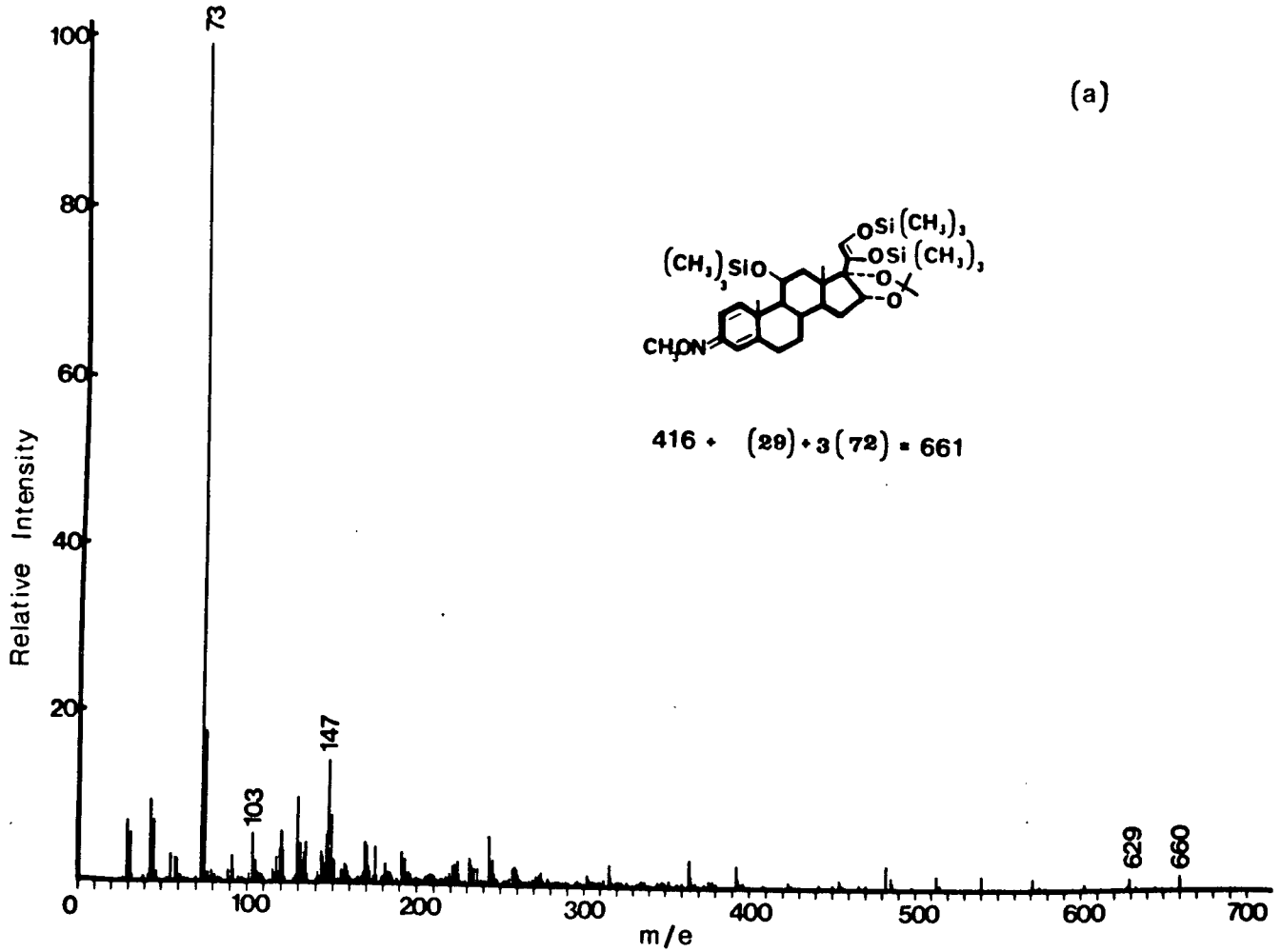


Figure 23-a : Electron-impact mass spectrum of $(MO)_1$ - $(TMS)_3$ - DSN

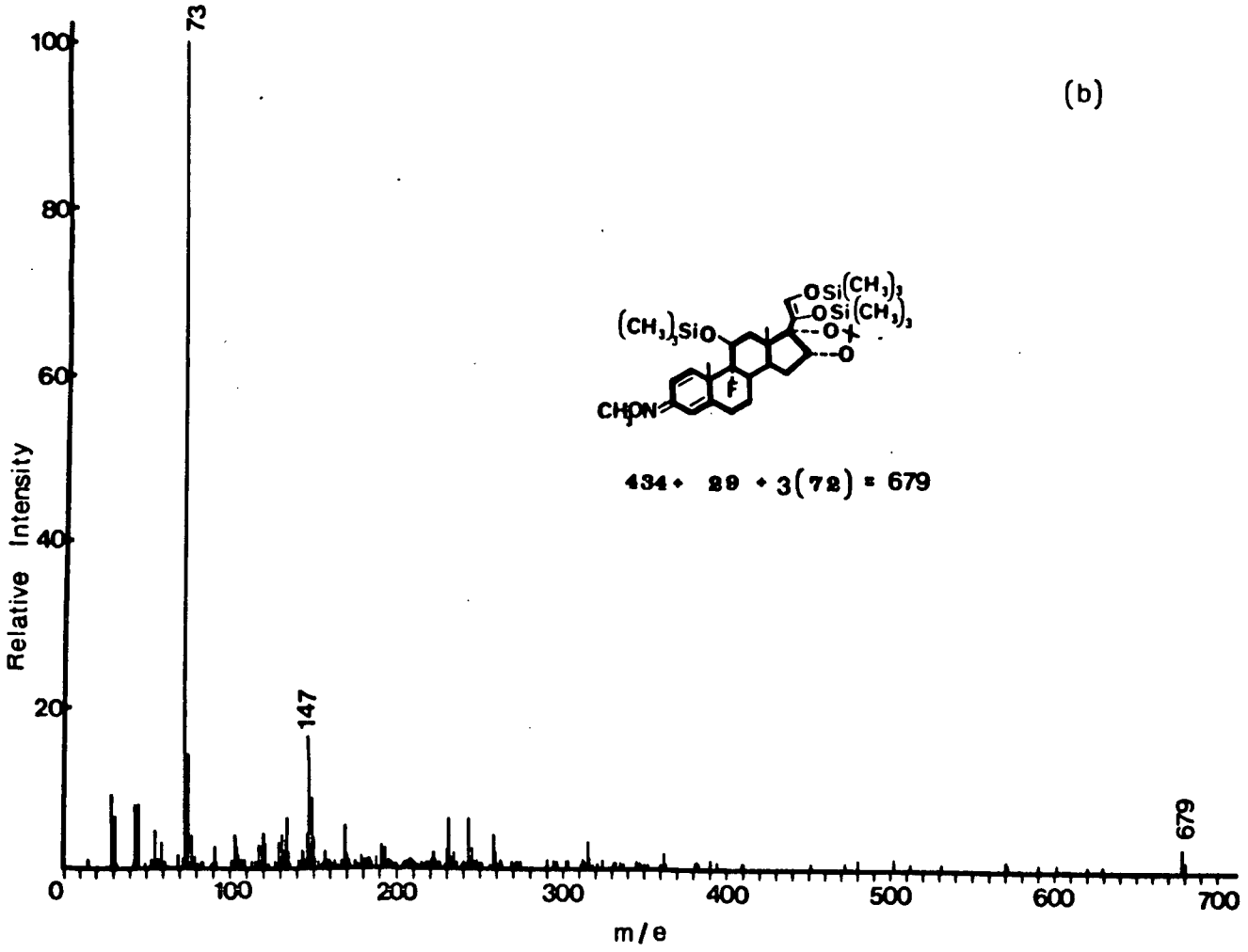


Figure 23-b : Electron-impact mass spectrum of $(MO)_1$ - $(TMS)_3$ - TA

tization reaction time led to an increase in the peak A at expense of the magnitude of peak B, suggesting the formation of A from B.

The fact that the chromatographic peaks from OV-7 columns represent two derivatization products of TA and DSN, however, did not interfere with the assay, because the contributions by the $(TMS)_3$-derivatives were less than 10% of the area of the peaks and, moreover, were in nearly constant ratio to those of the major $(TMS)_2$-derivatives. It is important that the derivatization reaction time is kept at 30 minutes and light be excluded.

TA shared all the previously mentioned characteristics of derivatization and gave $(MO)_1$-$(TMS)_2$-TA, m/e = 607 ($m^{+\cdot}$) as the major product and $(MO)_1$-$(TMS)_3$-TA, m/e = 679 ($m^{+\cdot}$) as the minor product (Figure 22-c and 23-b).

The presence of antibiotics in the medium showed no interference on the chromatograms.

(8) Calibration curves

The calibration curves for HC, TA and DSN extracted from medium containing 10% FCS were constructed. The curves were linear within the ranges studied, namely, 40-1600 ng/μL derivatized solution for HC (equivalent to 1.6 - 65.8 μg/mL medium), 60-1600 ng/μL derivatized solution for TA (equivalent to 2.4-63.7 μg/mL medium) and 20-300 ng/μL derivatized solution for DSN (equivalent to 0.8-12.0 μg/mL medium) (Table VII, VIII and IX).

Two calibration curves of HC were determined with a time lapse of six months between them. The relative standard deviation in slope values (0.905 and 0.946) was 3.1%. The combined data gave a straight line of $y = 0.947\ x - 0.005$ with $r^2 = 0.998$ where y = peak area ratio,

Table VII Estimation of HC after extraction from spiked medium samples

Amount added to 5 mL FCS-DMEM, µg	Weight ratio[a],x	Mean peak area ratio[b],y	Amount recovered, µg[c]	Mean recovery %
March/78				
16.45	0.135	0.105 ± 0.010[d]	13.95	84.82 ± 6.86[d]
24.67	0.203	0.144 ± 0.002	18.69	75.75 ± 1.00
41.42	0.337	0.280 ± 0.020	34.86	84.77 ± 5.84
61.68	0.506	0.439 ± 0.028	53.75	87.15 ± 5.36
164.48	1.348	1.193 ± 0.052	143.38	87.17 ± 3.71
Aug./78				
8.22	0.067	0.057 ± 0.001	7.92	97.51 ± 5.52
16.45	0.134	0.141 ± 0.008	18.39	111.82 ± 5.54
24.67	0.200	0.180 ± 0.001	21.65	90.16 ± 4.39
41.12	0.334	0.355 ± 0.022	44.11	107.28 ± 6.48
61.68	0.501	0.492 ± 0.024	60.55	98.17 ± 4.63
82.24	0.668	0.649 ± 0.021	79.34	96.47 ± 3.08
164.48	1.335	1.312 ± 0.020	158.93	96.63 ± 1.42
328.96	2.670	2.533 ± 0.047	305.40	92.84 ± 1.69

(a) Weight of HC/ weight of PRG

(b) Area of the peak of derivatized HC/area of the peak of derivatized PRG.

(c) Calculated from the standard curve of HC without extraction, $y = mx + c$, where $m = 1.026$ and $c = -0.009$, $r^2 = 0.999$, where r^2 = coefficient of determination, $y = mx + c$, where $m = 0.947$ and $c = -0.005$, $r^2 = 0.998$ for extracted samples, as plotted mean area ratio vs weight ratio.
The average mean recovery was 93.12 ± 9.74%.

(d) Mean ± S.D., calculated from nine measurements of three series of samples

Table VIII Estimation of TA after extraction from spiked medium samples

Amount added to 5 mL FCS-DMEM, µg	Weight ratio[a],x	Mean peak area ratio[b],y	Amount recovered, µg[c]	Mean recovery %
April/78				
25.92	0.142	0.073 ± 0.013[d]	22.75	87.75 ± 11.09[d]
39.00	0.214	0.123 ± 0.022	34.95	89.62 ± 12.36
64.80	0.354	0.214 ± 0.009	57.13	88.17 ± 3.33
86.40	0.531	0.292 ± 0.022	75.99	87.96 ± 6.15
129.13	0.708	0.408 ± 0.031	104.23	80.65 ± 4.37
257.70	1.412	0.805 ± 0.035	220.53	77.82 ± 2.49
Oct./78				
11.94	0.097	0.051 ± 0.003	11.79	98.76 ± 4.25
15.92	0.129	0.070 ± 0.001	14.84	93.21 ± 1.24
23.88	0.194	0.103 ± 0.011	20.35	85.20 ± 7.82
39.80	0.323	0.178 ± 0.001	32.60	81.92 ± 0.20
59.70	0.485	0.278 ± 0.008	48.98	82.04 ± 2.08
79.60	0.646	0.363 ± 0.005	62.92	79.05 ± 1.09
159.20	1.292	0.750 ± 0.028	126.29	79.33 ± 2.90
318.40	2.584	1.551 ± 0.036	257.58	80.90 ± 1.83
Feb./79				
11.94	0.119	0.066 ± 0.004	11.63	97.37 ± 4.90
15.92	0.159	0.093 ± 0.006	15.27	95.93 ± 4.95
23.88	0.234	0.144 ± 0.011	21.99	92.08 ± 6.21
39.80	0.396	0.233 ± 0.003	33.94	85.28 ± 0.91

(continued...)

Table VIII (continued)

59.70	0.595	0.356 ± 0.025	50.32	84.28 ± 5.66
79.60	0.793	0.508 ± 0.035	70.69	88.81 ± 5.86
159.20	1.586	0.913 ± 0.096	124.72	73.34 ± 8.07
318.40	3.171	1.640 ± 0.004	221.88	69.69 ± 0.17

(a) Weight of TA/Weight of PRG

(b) Area of the peak of derivatized TA/Area of the peak of derivatized PRG

(c) Calculated from the standard curve of TA without extraction, $y = mx + c$, where $m = 0.750$ and $c = -0.021$, $r^2 = 0.990$, where r^2 = coefficient of determination. $y = mx + c$, where $m = 0.533$ and $c = 0.012$, $r^2 = 0.996$ for extracted samples, as plotted mean area ratio vs weight ratio. The average mean recovery was 85.64 ± 7.19%.

(d) Mean ± S.D., calculated from nine measurements of three series of samples.

Table IX Estimation of DSN after extraction from spiked medium samples

Amount added to 5 mL FCS-DMEM, µg	Weight ratio[(a)],x	Mean peak area ratio[(b)],y	Amount recovered, µg[(c)]	Mean recovery %
4.0	0.079	0.040 ± 0.003	3.95	98.77 ± 4.72
8.0	0.163	0.106 ± 0.006	8.15	101.97 ± 4.70
12.0	0.242	0.169 ± 0.006	12.12	101.03 ± 3.01
16.0	0.325	0.236 ± 0.005	16.32	102.03 ± 1.81
24.0	0.468	0.350 ± 0.008	23.49	97.91 ± 2.05
40.0	0.726	0.555 ± 0.026	36.42	91.04 ± 4.03
60.0	1.092	0.847 ± 0.091	54.81	91.36 ± 9.53

(a) Weight of DSN/Weight of PRG

(b) Area of the peak of derivatized DSN/Area of the peak of derivatized PRG.

(c) Calculated from the standard curve of DSN without extraction, $y = mx + c$, where $m = 0.790$ and $c = -0.023$, $r^2 = 0.999$, where r^2 = coefficient of determination. $y = mx + c$, where $m = 0.713$ and $c = -0.004$, $r^2 = 0.999$ for extracted samples as plotted mean area ratio vs weight ratio. The average mean recovery was 97.73 ± 4.72%.

(d) Mean ± S.D., calculated from nine measurements of three series of samples.

x = weight ratio and r^2 is the coefficient of determination (Winer, 1971), indicating a satisfactory reproducibility of the assay.

Three calibration curves of TA were determined within a year. The relative standard deviation of slope values (0.515, 0.599 and 0.579) was 7.6%. The combined data gave a straight line of y = 0.553 x + 0.012 with r^2 = 0.996, where y = peak area ratio, x = weight ratio and r^2 is the coefficient of determination. The satisfactory reproducibility of the assay confirmed the validity of using the sum of double-peak areas, $(MO)_1$-$(TMS)_2$-TA and $(MO)_1$-$(TMS)_3$-TA for assay based on the assumption that the yield ratio of these two peaks was a constant.

The recoveries of three glucocorticoids added in various amounts were also tabulated (Tables VII-IX). The average mean recoveries ± S.D. were 93.12 ± 9.74%, 85.64 ± 7.19% and 97.73 ± 4.72% for HC, TA and DSN respectively.

The amounts of glucocorticoids extracted for the preparation of the calibration curves are large compared to what is needed for the activity of potent glucocorticoids, e.g., TA in cell cultures. Therefore, the entire volume of 10 mL of the culture medium or the pooled media of several plates had to be extracted in order to obtain enough TA for assay. On the other hand, the amounts used for calibration curves are realistic for assays of stock solution concentrates and similar solutions of pharmaceutical interest.

(9) Biological data

The applicability of the reported assay was demonstrated by determining the HC, TA and DSN levels in FCS-containing cell culture medium after various time periods of incubation with cultured human or mouse dermal fibroblasts. The results were summarized in Table X, XI and XII.

Table X Levels of HC in media as a function of time after incubation with cultured human dermal fibroblasts

Incubation time, hr.	n	Concentration assayed, µg/mL medium		
		A-2[(a)]	(% remaining intact)	A-4[(b)]
0	2	0.731 ± 0.134	(100.0 ± 18.3)[(c)]	-
0.25	1			0.462
2	2			0.392 ± 0.005
4	2	0.389 ± 0.035	(53.3 ± 4.7)	0.519 ± 0.009
6	2			0.474 ± 0.009
8	2			0.434 ± 0.016
12	2			0.436 ± 0.013
24	2	0.386 ± 0.044	(52.9 ± 6.0)	0.346 (n = 1)
48	2			0.455 ± 0.050

(a) Human dermal fibroblasts, F.H. passage 6.

(b) Human dermal fibroblasts, W.P. passage 4.

(c) The concentration of HC recovered from medium containing no cells was defined as 100% of intact drug.

* data given as mean ± S.D.

Table XI Levels of TA in media as a function of time after incubation with cultured mouse L-929 dermal fibroblast

Incubation time, hr.	n	Concentration assayed, µg/mL medium	Intact TA remaining in media, %
0	5	1.265 ± 0.082	100.0 ± 6.5[(a)]
2	2	1.032 ± 0.003	81.6 ± 0.2
4	2	1.025 ± 0.001	81.0 ± 0.1
8	2	1.030 ± 0.010	81.5 ± 0.8
11	2	1.011 ± 0.005	79.9 ± 0.4
24	2	1.060 ± 0.005	83.8 ± 0.4
48	1	1.011	79.9
72	2	1.028 ± 0.002	81.3 ± 0.1
83.5	2	1.079 ± 0.030	85.3 ± 2.4
94	2	0.919 ± 0.022	72.7 ± 1.7

(a) The concentration of TA recovered from medium containing no cells was defined as 100% of intact drug.

* data given as mean ± S.D.

Table XII Levels of DSN in media as a function of time after incubation with cultured mouse L-929 dermal fibroblasts

Incubation time, hr.	n	Concentration assayed, µg/mL medium	Intact DSN remaining in media, %
0	4	0.607 ± 0.012	100.0 ± 2.0[(a)]
4	2	0.514 ± 0.101	84.6 ±11.7
24	2	0.496 ± 0.033	81.7 ± 5.4

(a) The concentration of DSN recovered from medium containing no cells was defined as 100% of intact drug.

* data given as mean ± S.D.

Prolonged incubation of TA with cultured mouse L-929 dermal fibroblasts up to 83.5 hours did not cause appreciable decrease of intact TA in media. About 20% of TA was removed from the culture media by the mechanisms of absorption and/or adsorption by cells two hours after the TA administration. The levels of TA at various incubation periods between 2 hours and 83.5 hours did not vary appreciably. The significant decrease of TA level after 94 hours was observed, but unexplained.

To eliminate the possibility of decomposition, the stability of TA in medium upon incubation was studied at 37°C in the absence of cells for 72 and 96 hours. There was no appreciable decomposition due to the incubation at 37°C.

The levels of DSN after 4 and 24 hours of incubation with mouse L-929 fibroblasts also indicated about 20% loss of DSN from the medium.

Therefore, for any biological effect of synthetic glucocorticoids observed at the dose level of 1 μg/mL medium, the true bioavailable dose was probably not more than 0.2 μg/mL medium.

The levels of HC as a function of time had been measured twice at various intervals. The loss of HC from the media due to cells was larger than that of synthetic glucocorticoids. The HC also had rather constant levels as incubated with human dermal fibroblasts between 0.25 and 48 hours.

(10) Metabolites

No extra peaks were observed in samples incubated with cells for 0-108 hours and no appreciable change in the magnitudes of endogenous peaks in the mixture due to medium and 10% serum. It was proposed that no metabolite was apparently present unless its MO-TMS derivative had the same retention time of the derivatized parent compound, or that the amounts of metabolites were too small to be detected.

(11) Applicability of MO-TMS reaction to other glucocorticoids

Another glucocorticoid, diflorasone diacetate, was derivatized under the same reaction conditions; however, the reaction product gave four peaks in GLC chromatogram indicating multiple derivatizing products presumably due to the hydrolysis of acetates by hydrochloride generated in the reaction with methoxyamine hydrochloride and subsequent silylations at C_{17} and/or C_{21} free hydroxyl groups.

Therefore, diflorasone diacetate and probably other ester-group containing glucocorticoids, are not amenable to the GLC analysis using the subsequent derivatization with methoxyamine hydrochloride and N-trimethylsilyl imidazole.

Prednisolone could be derivatized to yield the $(MO)_2$ - $(TMS)_3$ - prednisolone derivative with the same reaction procedure.

Section II. Investigation of cross-linking of DNA induced by glucocorticoids and nuclear retention of TA

Materials and Methods

The descriptions of chemicals, reagents and apparatus used are compiled in Appendices I and II.

(A) Cell cultures and glucocorticoids

(1) Cell cultures

(a) Characteristics of cell lines

Human dermal fibroblasts are isolated by us from explants of biopsies from 25-35 year old males, stored at -193°C in liquid nitrogen. The cells were grown between 4th and 8th subculture in monolayer cultures to confluence in plastic culture dishes.

Mouse L-929 skin fibroblasts were derived from a chemically transformed clone in 1943 (Earle, 1943) and have since been maintained by Flow Laboratories. This cell line was recommended for assays of topical glucocorticoids (Berliner, 1967a), but differed from diploid human skin fibroblasts in important ways. L-929 cells are aneuploid, having an increased but variable chromosone count, 63 to 69 and they are not cell-density inhibited. Actually, they have been long considered mistakenly to be equivalent to human fibroblasts in response to topical glucocorticoids. Their growth rates are inhibited by glucocorticoids, while growth rates of human skin fibroblasts under identical experimental conditions are stimulated (Runikis et al., 1978; Thrash et al., 1974).

(b) Maintenance of cultured cells

Both cultured mouse L-929 and human dermal fibroblasts were maintained in Dulbecco's Modified Eagle Medium, pH 6.8 (DMEM) supplemented with 10% fetal calf serum (FCS), sodium bicarbonate 3.7 g/litre medium,

and the antibiotics, penicillin 100 units/mL, streptomycin 100 µg/mL and amphotericin B (Fungizone) 0.25 µg/mL. The cells were incubated at 37°C in 5% (v/v) CO_2 - 95% (v/v) air. The medium was changed twice weekly. The cellular morphology was examined regularly. Any plate with signs of microbial contamination, cellular degeneration or poor growth was discarded. Plates of about 4×10^6 cells per plate were used for studies. Usually, 5 plates were pooled for a single sample unless otherwise specified.

(2) Treatment with glucocorticoids

(a) Preparation of glucocorticoid stock solutions

Glucocorticoids investigated were HC and TA.

Stock solutions of the glucocorticoids, 10^3 µg/mL in propylene glycol vehicle, were prepared using the method of Brotherton (1971). Control solutions of the propylene glycol vehicle were prepared in a similar fashion. The procedure of solution preparation was as follows:

Solution A contained 50% of propylene glycol in methanol. Solution B contained 10^3 µg/mL glucocorticoid in methanol. The stock glucocorticoid solution, 10^3 µg/mL in propylene glycol, was obtained by mixing two volumes of solution A with one volume of solution B in a 500 mL vacuum flask, followed by the removal of methanol under vacuum. The control solution was prepared by mixing two volumes of solution A with one volume of pure methanol. After the removal of the methanol, the resultant control and glucocorticoid solutions were transferred to sterile amber bottles of 60 mL capacity and stored at -4°C in a cold room.

The stock solutions thus prepared were assayed by GLC-FID analysis before use to confirm the concentration and stability of the solution.

Immediately prior to incubation, the glucocorticoid stock solution was diluted to the desired concentration with the culture medium.

Control cultures received equivalent volumes of stock vehicles. The propylene glycol content of the media thus prepared did not interfere with cell growth.

(b) Incubation of cells with glucocorticoids

The 90% confluent cultures, approximately four days after seeding with 5 x 10^5 cells per plate, were incubated in glucocorticoid-containing media at 37°C. The concentrations investigated ranged from 10^{-8} M (4.4 ng/mL DMEM) to 5.01 x 10^{-6} M (2.2 µg/mL DMEM) for TA, from 2.76 x 10^{-6} M (1.0 µg/mL DMEM) to 2.76 x 10^{-5} M (10 µg/mL DMEM) for HC, and 2.40 x 10^{-6} M (1.0 µg/mL DMEM) for DSN. The incubation was maintained for varying periods up to 108 hr. At the end of the experiment, the glucocorticoid-containing media were removed for further testing and the cells were harvested with the aid of rubber policemen for various studies.

(B) Isolation and purification of DNA

(1) Homogenization of cells

The homogenization solution was composed of 8 M urea, 1 mM EDTA and 1% (w/v) S.D.S. in 0.24 M sodium phosphate buffer. The pH of the solution was adjusted to pH 6.8. The solution was stored at 4°C for no longer than three months (Meinke et al., 1974; DuVivier et al., 1978).

Ten mL of homogenization solution were distributed among plates, and the solution was left in contact with cells for 5 min. at 4°C. The cell suspension was transferred with the aid of a rubber policeman into a glass-Teflon homogenizer. There it was manually homogenized with 10 to 20 strokes until an uniformly viscous homogenate was obtained.

(2) Deproteinization

This homogenate was transferred into an Erlenmeyer flask where

it was brought up to 1 N NaCl by adding 585 mg of NaCl(s) to facilitate the dissociation of chromosomal proteins from the chromatin (Meneghini, 1976). The deproteinization was done by gentle shaking with six volumes of chloroform/butanol (4:1, v/v), and the aqueous phase was separated by centrifugation. The deproteinization of the aqueous phase was repeated two times with fresh chloroform/butanol mixture.

(3) Chromatography on hydroxyapatite column

(a) Preparation of the column

Fours grams of hydroxyapatite powder (HAP) were hydrated in 50 mL of 0.01 M phosphate buffer, pH 6.8, with gentle swirling (when hydrated, Bio-gel HAP swelled to 2-3 mL/g dry wt.) The suspension was boiled in a water bath for 30 min. and allowed to settle for 15 min. The fines in the cloudy upper level and at the top of the settled gel were decanted. The paste was resuspended in 20 mL of the same buffer for column pouring.

The column was packed in a 2 cm (i.d.) x 10 cm glass column by wet pouring technique. The set-up of the HAP chromatography consisted of a gradient mixer, a peristaltic pump and a fraction collector in addition to the packed column.

(b) Isolation of DNA by chromatography

The combined deproteinized aqueous phase was applied on the top of the packed hydroxyapatite column and chromatographed with a linear gradient of 0.01 M to 0.5 M of phosphate buffer. The flow rate of the eluent was controlled to about 30 mL/hr/cm^2 by the peristaltic pump. Bernardi (1971a) recommended flow rates of 5 - 50 mL/hr/cm^2 to avoid the distortion of chromatographic peaks.

The fractions of 3.2 mL each were monitored by measuring the absorbance at 260 nm (A_{260}), and fractions containing DNA were pooled.

(4) Concentration of the pooled DNA by Amicon filtration

The stirring filtration unit (Model 12, Amicon) was used with prehydrated Diaflo XM 100A membranes (Amicon), which retain molecules of size larger than 100,000.

The solution was introduced into the transparent sleeve with the aid of a 5 mL syringe before applying positive pressure with high-purity nitrogen. The solvent and any molecule having M.W. smaller than the pore size passed through the membrane, leaving the DNA in retentate. Two aliquots of 5 mL of dilute saline-citrate (DSC, 0.015 M NaCl and 0.0015 M Na_2-citrate pH 6.85) were added to wash the retentate for removal of any phosphate ions and other cations. After the wash, the membrane was soaked in a test tube with 5 mL of DSC overnight at $4^{o}C$ in order to ensure the maximum recovery of DNA from the retentate and the membrane (Bohnert, 1978).

(C) Burton's diphenylamine assay for DNA

A modified version of Burton's assay (1955) was employed, in which the sensitivity of the assay was improved by adding acetaldehyde to the reagents and by allowing the solution to stand for 16-20 hr. at $30^{o}C$ instead of heating it at $100^{o}C$ (Burton, 1956).

Diphenylamine reagent reacts with the deoxyribose moieties which are bound to purines in DNA and forms blue-colored complex. DNA can therefore be quantitated colorimetrically by measuring the absorbance at 600 nm. Calibration curves were constructed within the concentration range of 2 to 50 µg/mL.

(1) Preparation of solutions:

DNA stock standard solution of 1 mg/mL: Calf thymus DNA was dissolved in 5 mM NaOH (Chandra and Appel, 1973). The solution could be

stored in refrigerator for about 6 months. The DNA working standard solution was prepared by mixing equal volumes of stock standard solution and 1 N $HClO_4$, and heating at 70°C for 15min. The solution was then cooled slowly at room temperature and diluted to the concentration of 100 µg/mL with 0.5 N $HClO_4$ for constructing the calibration curve. The solution could be stored in refrigerator for 3 weeks.

Diphenylamine reagent, 1.5% (w/v), was prepared by dissolving 1.5 gm of diphenylamine in 100 mL acetic acid and 1.5 mL concentrated H_2SO_4. Immediately prior to use, 0.1 mL of aqueous acetaldehyde, 16 mg/mL, was added for each 20 mL of reagent.

(2) Procedure

a. the working standard or test solutions, 0.2 to 1.0 mL, were taken, and the volumes were made up to 1.0 mL with 0.5 N $HClO_4$.

b. the resulting DNA solution was mixed with 2 volumes of diphenylamine solution containing acetaldehyde, and allowed to stand for 16-20 hr. at 30°C.

c. The absorbance at 600 nm was measured. Calibration was repeated for each assay.

(D) Bio-Rad protein assay

The Bio-Rad protein assay is a dye-binding assay based on the differential color change of the Coomassie Brilliant Blue G-250, in response to its binding to various concentrations of protein (Bradford, 1976). When the binding occurs, the absorbance maximum for the acidic dye solution shifts from 465 nm to 595 nm (Reisner et al., 1975; Sedmak and Grossberg, 1977). By monitoring the absorbance at 595 nm, the protein concentrations can be determined.

(1) Selection of protein standard

The best protein standard is a purified preparation of the protein being assayed; however, the identity of the protein was not known in this research, and therefore was not available. Of the two commercially available standards, bovine serum albumin was used instead of bovine gamma globulin, because bovine serum albumin gave a color yield closer to non-histone and histone proteins-containing samples than the globulin standard does. An example of the suitability of albumin as a standard for assaying a complex protein mixture (adrenal gland subcellular fractions) has been given by Polland et al. (1978).

(2) Procedure

The following microassay procedure, suitable for amounts of protein ranging from 1 to 20 μg, was employed (Bio-Rad bulletin, 1977).

A standard curve was prepared each time the assay was performed. A series of dilutions of protein standards containing from 1 to 25 μg/mL in 0.8 mL of blank solvent, identical to that of the test sample, were placed in clean, dry test tubes. Same volume of sample solvent served as blank. Dye reagent concentrate, 0.2 mL, was added, followed by a gentle vortex avoiding excess foaming. A_{595} were measured against reagent blank 5 minutes after the addition of the dye reagent and A_{595} was plotted against the amount of protein.

(E) Thermal scanning analysis, T_m and hyperchromicity

(1) Denaturation profile

Four cuvettes containing 0.25 mL of the solvent, dilute saline-citrate (DSC), and three test DNA samples respectively, were heated from 30°C to 90°C with an increment of 1°C/min. The cuvettes were scanned 20 seconds per cycle. The A_{260} of each sample was recorded as

a function of temperature to generate the denaturation profile (Figure 24).

(2) Renaturation profile

The temperature of the cuvettes were brought down rapidly from 90°C to 50°C within 2 minutes controlled with the thermoprogrammer after the completion of thermal denaturation. The temperature was allowed to stay at 50°C for 10 minutes, then a further cooling to 10°C was conducted in an identical fashion. The A_{260}'s were similarly plotted as a function of temperature to generate the renaturation profile (Figure 24).

(3) Data analysis

Denaturation profile: The melting points of the DNA samples were calculated from the denaturation profile as shown in Figure 24, based on the definition of T_m as that temperature at which half of the absorbance increase is achieved. Besides T_m, the hyperchromicity was also measured as the ratio of total absorbance change to the initial absorbance before denaturation, $(\Delta A_{260})_{50^\circ \longrightarrow 90^\circ} / (A_{260})_{50^\circ}$, expressed in percentage, to characterize the denaturation profile.

Renaturation profile: The hypochromic effect of renaturation due to cooling was measured. The % renaturation was calculated as $\left((\Delta A_{260})_{90^\circ \longrightarrow 50^\circ} / (\Delta A_{260})_{50^\circ \longrightarrow 90^\circ}\right) \times 100\%$ for cooling to 50°C and $\left((\Delta A_{260})_{90^\circ \longrightarrow 10^\circ} / (\Delta A_{260})_{50^\circ \longrightarrow 90^\circ}\right) \times 100\%$ for cooling to 10°C.

(F) UV-A irradiation of cultured fibroblasts

Human and L-929 fibroblast were irradiated in the plates where they were grown to 90% confluency. Immediately before irradiation, the culture media in the plates were replaced with PBS or PBS plus test com-

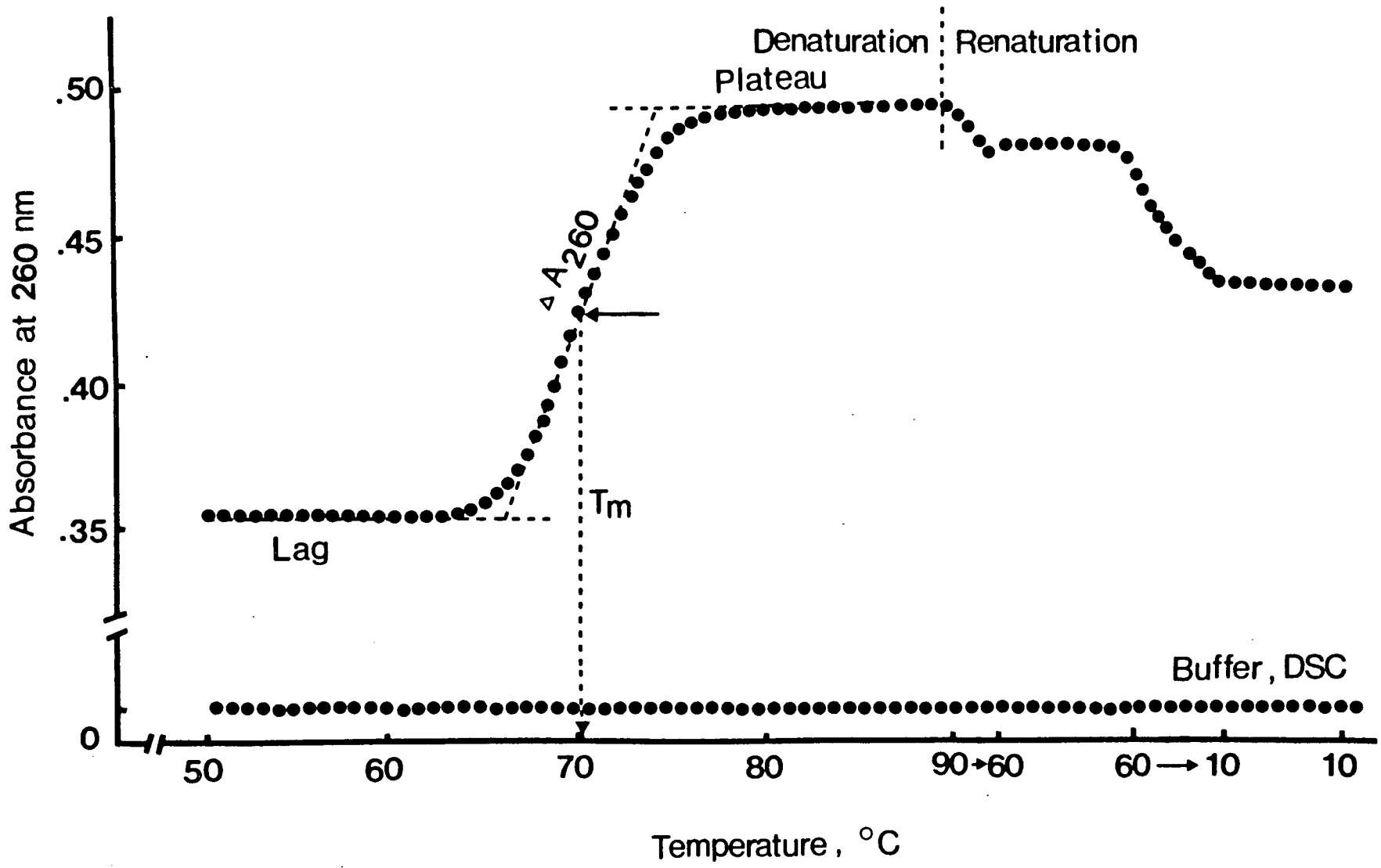


Figure 24 : Typical denaturation - renaturation profile of DNA isolated from cultured fibroblasts

pound (glucocorticoids or 8-methoxypsoralen) to about 0.15 cm in depth (8 ml/plate). The plates were kept from light by wrapping them with aluminum foil until placed in the irradiation incubator, where they were shaken slowly (100 strokes/hr) with their lids on, at 30°C.

The incubator was equipped with black light blue fluorescence lamps emitting light within the range of 310 nm to 360 nm, with most of the energy flux at 330 nm which reached the cells at an intensity of 0.6 mW/cm^2. Varying irradiation periods of time were used.

The cells were examined for the influence of the irradiation on their viability. They were examined under microscope for persistence of their attachment to the plates. Then, 0.4% trypan blue was allowed to contact the cells for 1 minute. After rinsing 5 times with PBS, the cells were re-examined for staining. Since only dead cells stain, the proportion of non-stained cells to stained cells provided an indication of cell viability.

(G) Isolation and solubilization of nuclei

All procedures were carried out at 4°C unless specified otherwise.

Two to five x 10^7 cells were incubated in DMEM with 10^{-8} M tritiated TA at 37°C in the absence or presence of 5 x 10^{-6} M unlabeled TA (Johnson et al., 1979a; Ishii et al., 1972; Garola and McGuire, 1977). At varous times, the reactions were stopped by replacing the DMEM in each plate with 1 mL of ice-cold Ca^{++}- and Mg^{++}-free PBS containing 5 x 10^{-6} M unlabeled TA. The samples then were washed three times with PBS by resuspension and centrifugation at 1,000 x g. Cells were lysed by 5 mL of 0.5% Triton X-100 in EDTA/saline containing 5 x 10^{-6} M unlabeled TA, to release cytoplasmic materials while leaving the

nuclei intact (Meneghini, 1976). To ensure the complete rupture of cytoplasmic membranes, the cell suspension was vortexed vigorously three times for 2 minutes each. The nuclei were collected by centrifugation at 1,000 x g for 10 minutes. The pellets were resuspended with PBS, and examined under the phase-contrast microscope at 150 X magnification (Hudson and Dimmock, 1977).

After the removal of PBS, the pellets were solubilized in 2.5 mL of hypotonic solubilizing buffer, pH 7.4, containing $CaCl_2$, $MgCl_2$, dithiothreitol and glycerol. The suspension was sheared manually 10 times with a syringe with 25 gauge 5/8 inch hypodermic needle whenever the homogeneity of the solubilized nuclei preparation was not satisfactory.

(H) Isolation of chromatin from isolated nuclei

All procedures were carried out at $4^{o}C$ unless specified otherwise.

The chromatin was prepared from intact isolated nuclei. The procedure of Johnson and Baxter (1978) was used with their modifications of conditions of washing and storage (Johnson et al., 1979b).

The chromatin was isolated by a stepwise lowering of the ionic strength. First, the nuclear pellet was resuspended in 10 mL of 80 mM NaCl, 20 mM EDTA, pH 6.3, and the extracted nuclear pellet was centrifuged at 5,000 x g for 10 minutes and the supernatant removed. The pellet was then resuspended in 10 mL of 1.5 mM NaCl, 0.15 mM Na_2-citrate (0.01 SSC) pH 7.0 and recentrifuged again. The purified chromatin, as a clear hydrated gel, was solubilized in a buffer containing 0.01 SSC and 25% (v/v) glycerol, and stored at $-20^{o}C$.

(I) Liquid scintillation counting

The counting efficiency, background counts and quench effects of the various solvents and cell components present in experiments of scintillation counting were as follows.

The counting efficiency of tritiated samples in 10 mL of Aquasol-2 using 50 mg of ^{3}H-toluene standard was 45.5 ± 0.5% (n=26). The background counts of various solvents, including DMEM (20 μL), PBS (100 μL), Triton X-100 with cytoplasm (100 μL), solubilizing buffer with nuclei or chromatin (200 μL) and 0.01-0.5 M Nap buffer (3.2ml each), in 10 mL of Aquasol-2 ranged from 21-29 cpm. Aquasol-2 also gave a background of 20 cpm. Thus, these solvents contributed no significant counts.

The quench effect of these various solvents with or without additives (cytoplasm, nuclei or chromatin) was negligible. The recoveries of radioactivity in these aliquots, measured with known amounts of ^{3}H-toluene standard ranged from 91 to 98%. Aquasol-2 formed single phase systems with these reagents. No quench correction therefore was applied because experimental variations due to other sources were considered to be at least 5%.

EXPERIMENTS

(A) Test for possible cross-linking of DNA induced by glucocorticoids

Tests for cross-linking of DNA were done with cells incubated with HC- or TA-containing DMEM. The concentrations were 1 μg/mL DMEM or 10 μg/mL DMEM for both glucocorticoids. The incubation time varied between 0 and 96 hr. In studies with UV-A irradiation, the cells were irradiated by long wavelength UV-A for 20 min, 1 hr or 2 hr prior to the harvest of cells.

The DNA was isolated from harvested cells using the procedure described in Materials and Methods subsection B, and its purity was examined by determining the A_{260}/A_{280} ratio, the amount of protein remaining with the DNA as well as the T_m and hyperchromicity. The amount of the isolated DNA was measured by Burton's diphenylamine assay or by estimation from A_{260}.

Two techniques were utilized to detect the presence of cross-linked DNA by glucocorticoids: (i) hydroxyapatite chromatography of DNA samples, which were thermally denatured at 95° for 5 min, followed by rapid cooling in ice-water bath for another 5 min, and (ii) thermal scanning analysis of native DNA samples for establishing the denaturation-renaturation profiles.

The DNA isolated from mouse L-929 fibroblasts, irradiated with UV-A in the presence of 8-methoxypsoralen (8-MOP), 5 μg/mL PBS, served as the positive control of cross-linked DNA to confirm that (i) the isolation procedures of DNA did not conceal the presence of cross-linking and (ii) the two techniques selected to detect the cross-linking, HAP chromatography and denaturation-renaturation kinetics, are sufficient to detect

cross-linking of DNA (Cech et al., 1979; Dall'Acqua et al., 1972; Cole, 1971).

(B) Retention of ^{3}H-TA during the isolation of DNA

This series of experiments identified steps in isolation procedures, if any, which removed ^{3}H-TA from nuclear DNA. It is sufficient to examine the steps of deproteinization and HAP chromatography.

The nuclei isolated from mouse L-929 fibroblasts, treated with ^{3}H-TA for 6 or 96 hours, were homogenized manually and deproteinized three times as described in Materials and Methods B-1 and B-2. The three $CHCl_3$/BuOH extracts were collected separately and evaporated to dryness under a nitrogen stream. The residues were dissolved in 1 mL of 0.1 N NaOH (Kobayashi, 1978) and 10 mL of Aquasol-2 was added for counting. The deproteinized DNA in aqueous phase was chromatographed as described in Materials and Methods B-3. Fractions collected were monitored for DNA with A_{260} measurement and for radioactivity with liquid scintillation counting by adding 3.2 mL of each fraction to 10 mL of Aquasol-2.

(C) Specific retention of ^{3}H-TA in whole nuclei of cultured mouse L-929 fibroblasts

The nuclear retention of ^{3}H-TA was determined by liquid scintillation counting. Triplicate samples of 200 µl aliquots of solubilized nuclei, obtained as described in Materials and Methods section G, were counted in vials of 10 mL of Aquasol-2. The radioactivity was normalized for nuclear protein determined by Bio-Rad protein assay, and for DNA determined by Burton's assay following the practices of Johnson and Baxter (1978).

The specifically bound TA was determined by the commonly accepted procedure of subtracting the nonspecific binding, determined in parallel incubations containing 5×10^{-6} M unlabeled TA, from the total (specific and non-specific) binding measured in the absence of unlabeled TA (Johnson et al., 1979a; Ishii et al., 1972; Garola and McQuire, 1977).

The data were converted from cpm/μg nuclear protein and cpm/μg DNA to fmole/μg nuclear protein and fmole/μg DNA, respectively, by multiplying with the factor of 3.296×10^{-2} fmole/cpm. This conversion made the data in units comparable with the literature data of cytoplasmic receptor binding.

(D) Specific retention of ^{3}H-TA in chromatin of cultured mouse L-929 fibroblasts

The specific retention of ^{3}H-TA was determined by liquid scintillation counting. Triplicate samples of 200 μL aliquots of solubilized chromatin, prepared as described in Materials and Methods subsection H, were counted in vials of 10 mL of Aquasol-2. The radioactivity was normalized for chromosomal protein determined by Bio-Rad protein assay, and for DNA determined by Burton's assay.

The specifically retained TA was determined by subtracting the nonspecifically retained TA, determined simultaneously in incubations containing 5×10^{-6} M unlabeled TA, from the total (specific and non-specific) binding, measured in the absence of unlabeled TA. The data were expressed as fmole/μg chromosomal protein and fmole/μg DNA. The specific retention of TA in chromatin was plotted against the duration of experiments.

(E) Subchromatin localization of TA

The HAP dissociation method, described by Bloom and Anderson (1978), fractionates chromosomal proteins in relation to their binding properties to DNA.

Hydroxyapatite powder, 2 gm, were prehydrated in 10 mM NaP buffer, pH 7.0. Columns as described previously (Materials and Methods B-3-a) were used. Chromatin samples were then eluted sequentially with 20 mL portions of the 11 solvents listed in Table V. The flow rates were kept at 30 mL/hr/cm^2 at 4°C. Fractions of 10 mL were collected.

The collected fractions were monitored for ^{3}H-TA by liquid scintillation counting of 1 mL of each fraction in 10 mL of Aquasol-2, and for dissociated proteins by measurements of absorbance at 230 nm (Bluthmann, 1977). The radioactivity and A_{230} were plotted against fraction numbers. The subchromatin localization of TA was indicated by these two plots.

RESULTS AND DISCUSSION

(A) Morphology of glucocorticoid-treated and glucocorticoid-free mouse L-929 dermal fibroblasts

Glucocorticoids induce definite morphological changes in fibroblasts (Figure 25). Glucocorticoid-treated L-929 fibroblasts were larger, flatter, less densely packed, and often more polygonal and epithelial in appearance than glucocorticoid-free controls. Such morphological changes have been discussed in detail by Pratt (1978), Rasche and Ulmer (1968) and Berliner (1967b), and were considered to represent a phenotypic reversion from a "transformed, or tumor cell-like state" to a more tightly regulated growth state (Wigler et al., 1975).

(B) Evaluation of cross-linking of DNA with glucocorticoids

(1) Quantity and quality of isolated DNA

The quantities of DNA isolated by techniques described in Materials and Methods subsection B from mouse L-929 and human dermal fibroblasts were measured using either Burton's assay or estimation from A_{260}. One absorbance unit at 260 nm was assumed to be equivalent to 50 µg of DNA/mL (DuVivier et al., 1976). Parish (1972) has suggested that one unit of A_{260} represents 66.7 µg of DNA/mL. The value of 50 µg/mL was used, however, since the latter value provided better agreement between the two techniques for DNA quantitation.

With human cells, the DNA ranged from 6.8 to 9.2 µg per 10^6 cells, which were comparable to the literature value, 8.30 ± 0.45 µg per 10^6 cells (Fujimoto et al., 1977). The amounts of DNA recovered from mouse L-929 fibroblasts were higher, from 8.4 to 17.5 µg per 10^6 cells. The higher DNA content of mouse L-929 fibroblasts can be explain-

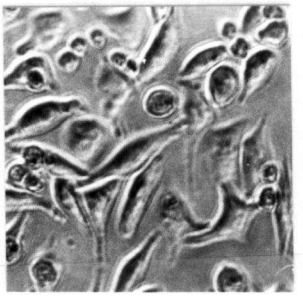
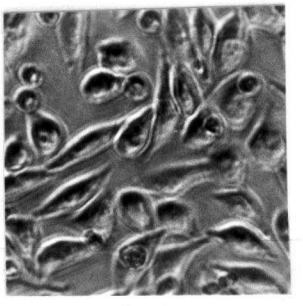
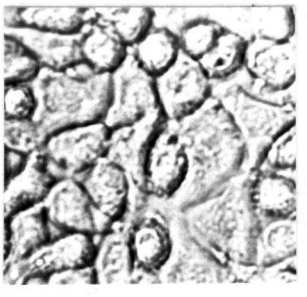
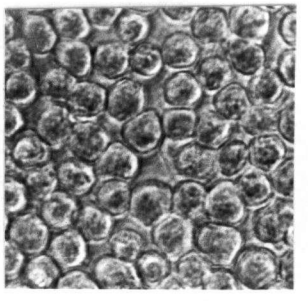
Incubation time (hr)	Glucocorticoid-treated	Glucocorticoid-free
0,2,4,6,24		
48,72,96		

Figure 25 : Morphology of glucocorticoid-treated and glucocorticoid-free mouse L-929 fibroblasts

ed by the aneuploidy of mouse L-929 cells, which have upwards of 65 chromosomes per cell compared to human fibroblasts with 46 chromosomes.

The purity was accepted as adequate for this research if the isolated DNA was as pure as commercial DNA (Sigma, type I). The latter is claimed by the manufacturer to be essentially free of RNA and protein, and is a widely used reference standard. It is not possible to isolate absolutely pure DNA from cells. The following three criteria were used.

(a) The ratio of absorbance, A_{260}/A_{280}

The reference standard of commercial DNA (Sigma, type I) gave a mean value ± S.D. of the ratio of A_{260}/A_{280}, 1.69 ± 0.03 (n = 83); therefore, the DNA isolated from cultured fibroblasts were acceptable if the ratio A_{260}/A_{280} was higher than 1.65. The literature criterion requiring a ratio higher than 1.80 (DuVivier et al., 1978) was not adopted for the above mentioned practical reason.

(b) Protein content:

DNA preparations containing less than 5.4% (w/w) of protein, measured by the Bio-Rad assay, were admissible for further studies. The usual criterion for "substantially" protein-free DNA is less than 1% (Meneghini, 1976; Parish, 1972). Since Sigma DNA showed also higher protein content (5.4%), we accept the result as artifactually high due to high blank values in relation to the small amounts of the protein found in DNA.

(c) T_m and hyperchromicity

The melting point, T_m, and the hyperchromicity value of the isolated DNA from cultured fibroblasts were examined.

The isolated DNA in DSC had a melting point of 71.2 ± 0.7°C (n = 9) and hyperchromicity of 30.0 ± 4.1% (n = 9). These values were comparable to those of the standard of calf thymus DNA, 70.3 ± 0.3°C (n =

5), and fell within the range of literature values (Marmur and Doty, 1962; Spodheim-Maurizot et al., 1979).

The melting curves of isolated DNA corresponded to those reported in literature for substantially pure DNA. The data of thermal scanning analysis was therefore accepted as evidence of adequate purity of the DNA, isolated by the techniques reported here, for the purpose of this project. The discrepancies observed between the A_{260}/A_{280} ratio as well as the protein values observed and the respective literature values were attributable to methodological limitations.

(2) Examination of cross-linking of DNA

(a) Hydroxyapatite chromatography

Hydroxyapatite chromatography was employed for three different purposes. Firstly, it was applied for isolating DNA from homogenate of cell lysate. Secondly, hydroxyapatite chromatography was adapted to distinguish denatured single-stranded DNA from native double-stranded or from renatured native-like double-stranded DNA. Thirdly, it was applied for fractionating chromosomal proteins selectively from chromatin, presented in Results and Discussion subsection F. Examination of cross-linking of DNA utilized the first two properties of hydroxyapatite to separate DNA from other components of cell lysate, in its native form, and to distinguish between its denatured single-stranded and renatured double-stranded forms when subjected to heat denaturation.

A linear concentration gradient instead of stepwise elution technique was employed, to avoid the artifactual "false peak", which has been reported to be associated with the stepwise elution technique (Tiselius et al., 1956; Hjerten, 1959). Sodium phosphate (NaP), which is slightly less effective than potassium phosphate (KP), was used as the eluting agent.

The DNA of deproteinized cell homogenates was well resolved from other cellular components, presumably RNA and protein, and eluted between 0.28 and 0.33 M sodium phosphate buffer, pH 6.8 (Figure 26).

The early peak of the chromatogram, eluted at 0.04 - 0.08 M sodium phosphate buffer, consisted of some unidentified A_{260}-absorbing species and homogenization buffer. Meinke et al. (1974) found these low salt fractions contained protein and sheared nucleic acids (RNA and/or DNA). Traces of chloroform/butanol left after the deproteinization step made no contribution to the magnitude of this peak.

Two peaks eluting between 0.12-0.25 M were unidentified. However, they were believed to be ribonucleic acid (RNA) on the basis of the reported chromatographic behaviour of RNA (Bernardi, 1971a).

The ability of hydroxyapatite to separate native double-stranded and denatured single-stranded DNA was demonstrated in Figure 27. The thermally denatured single-stranded DNA was eluted from hydroxyapatite columns at a sodium phosphate molarity of 0.18 - 0.23 M, distinctly lower than that of eluting native DNA, 0.27 - 0.31 M. The renatured as well as denatured but cross-linked DNA behaved identically to the native DNA and were seen to be eluted at the same molarity as native DNA, as expected from literature reports (Bernardi, 1969b).

The recovery of native DNA was quantitative, whereas that of denatured DNA was only 34-60%. This was expected due to the aggregation of the denatured DNA molecules caused by residual protein and/or intermolecular base pairing (Bernardi, 1971b).

The DNA isolated from (8-MOP)-treated and (UV-A)-irradiated mouse L-929 fibroblasts were split into two aliquots. One aliquot was thermally denatured at 95°C for 5 minutes followed by rapid cooling in an ice-water bath for another 5 minutes. The denatured aliquot and the

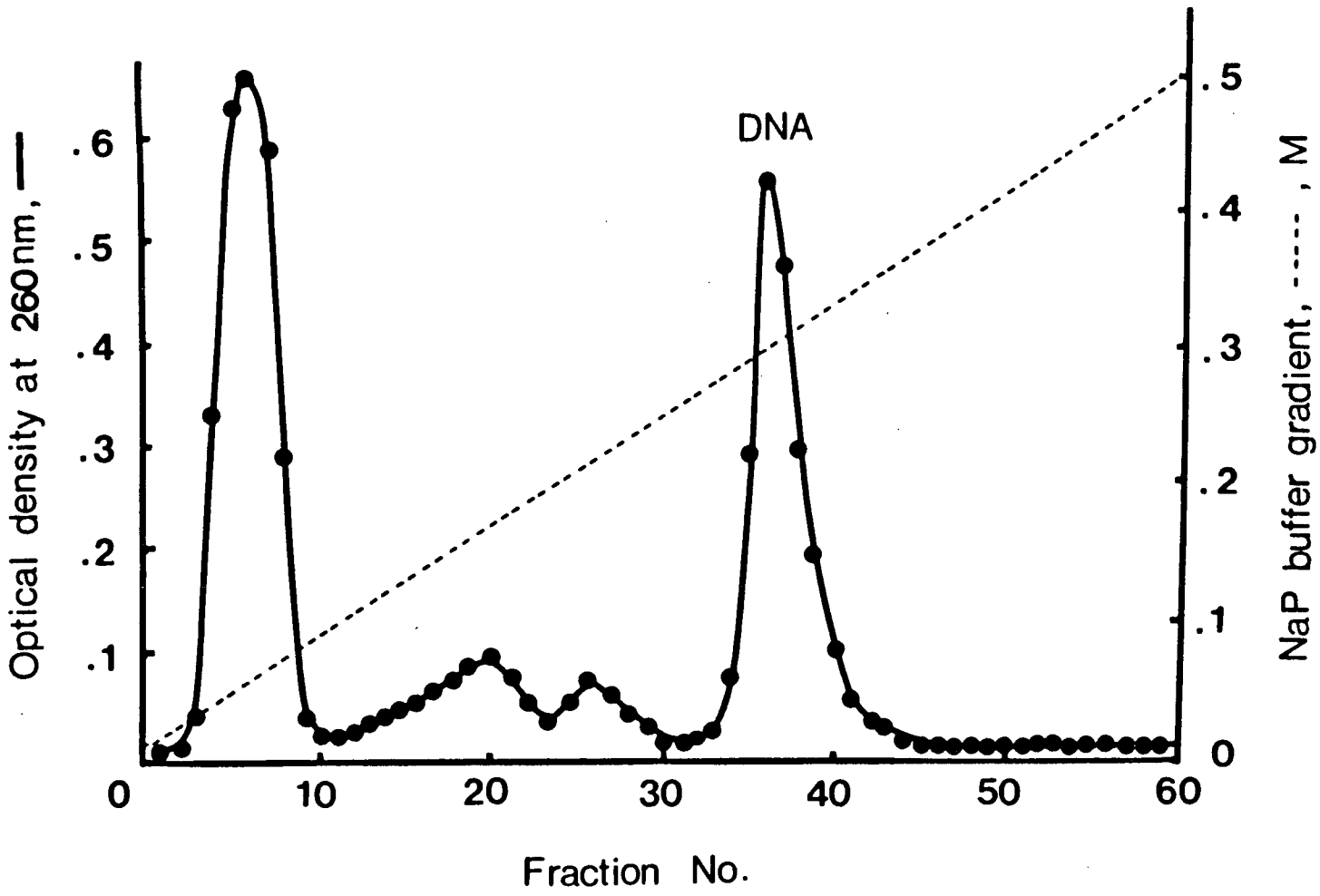


Figure 26 : Representative chromatogram of deproteinized cell homogenate applied to HAP column

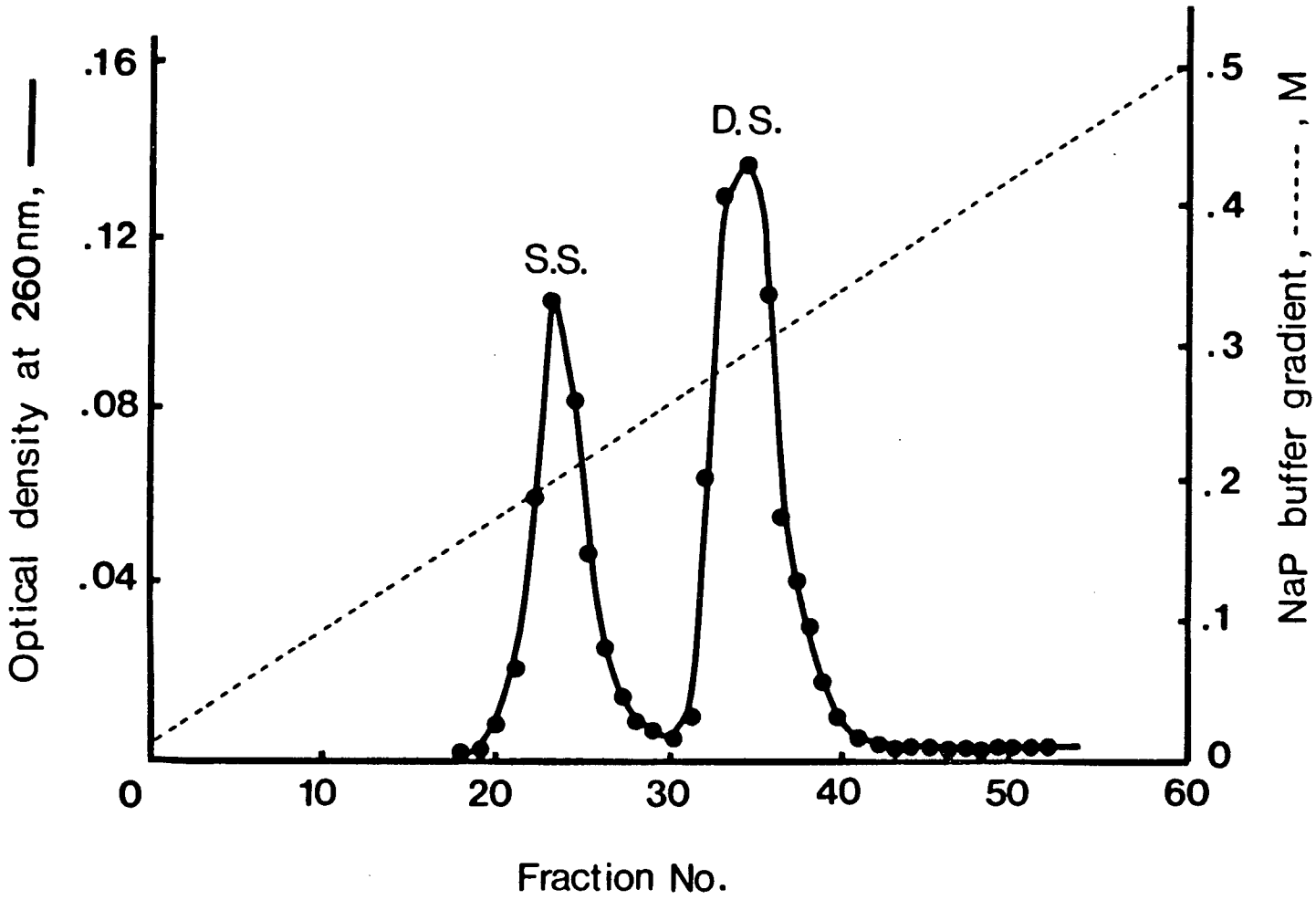


Figure 27 : Representative chromatogram of native and denatured DNA applied to HAP column

S.S. : single-stranded DNA D.S. : double-stranded DNA

native counterpart were eluted through the individual hydroxyapatite columns simultaneously.

The native DNA preparation contained only double-stranded DNA, while the denatured DNA consisted of both denatured single-stranded and renatured double-stranded DNA. The renatured double-stranded peak indicated the presence of cross-linked DNA. The magnitudes of the peak of the cross-linked DNA were similar in cases of UV-A irradiations from 20 minutes to two hours in the presence of 8-MOP. The hydroxyapatite chromatograms of native and denatured DNA isolated from (8-MOP)-UVA treated mouse L-929 fibroblasts (Figure 28), therefore, served as a positive control for detecting the presence of cross-linked DNA induced by glucocorticoid and glucocorticoid-UVA treatments.

The DNA isolated from hydrocortisone- or triamcinolone acetonide-treated mouse or human dermal fibroblasts, with or without UV irradiation, had no detectable double-stranded DNA remaining after the boil-cool denaturation. The glucocorticoid-treated cells were, therefore, unlikely to possess cross-linked DNA molecules at a detectable level. Twenty-three studies with various combinations of (1) type of glucocorticoid, hydrocortisone or triamcinolone acetonide, (2) concentration of glucocorticoid, 1 μg/mL or 10 μg/mL media, (3) cell line, human or mouse L-929 dermal fibroblast, (4) incubation period, 0,2,4,8,12,24,48,72,84,96 or 108 hours, and (5) with or without UV-A irradiation, as well as various periods of irradiation, 20 minutes, 1 hour or 2 hours, were conducted as summarized in Table XIII. However, the denatured DNA from all these studies showed only a single peak indicating that only single-stranded molecules were present. Therefore, hydroxyapatite chromatography showed no evidence for cross-linking induced by glucocorticoid or glucocorticoid-UVA treatments (Figure 29).

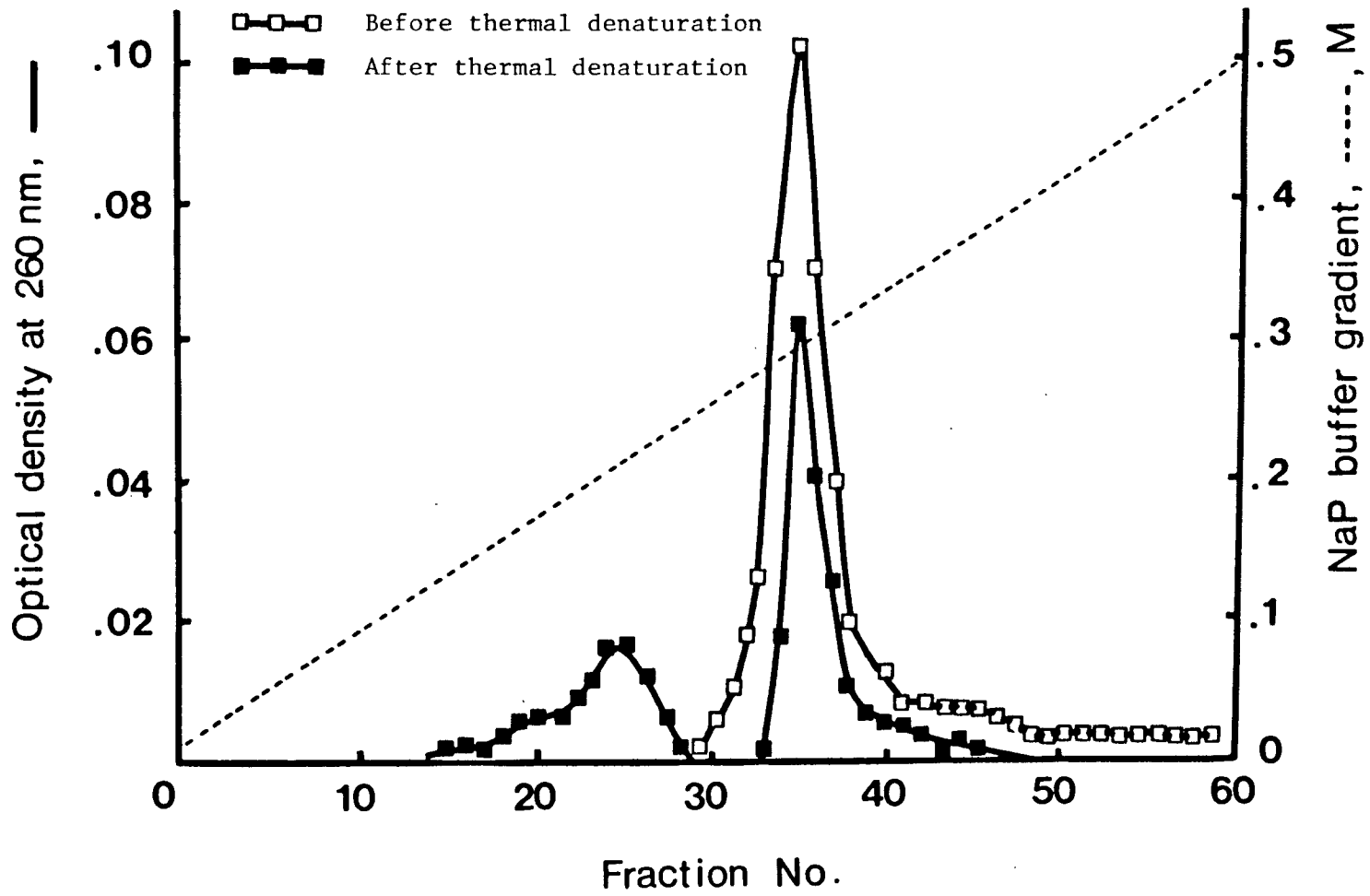


Figure 28 : HAP chromatography of DNA isolated from (8-MOP)-treated and UV-A irradiated mouse L-929 fibroblasts

Table XIII Conditions examined to investigate the formation of cross-linked DNA by glucocorticoids(*)

Cell line	Glucocorticoid	Concentration µg/mL media	Incubation time, hr.	UV-A irradiation interval, hr.
L-929	HC	10	0,2,4,8,12,24,48,72, 96	2
		1	4, 12,24, 96	-
	TA	1	0,2,4,8,12,24,48, 96	-
		10	96	-
		10	0	1/3, 1, 2
		10	24, 96	2
Human	HC	1	4, 12,24,48,72,84,96	-
	TA	1	0,2,4,8,12,24,48,72,84,96, 108	-

(*) : Twenty-three independent experiments were carried out with various combinations of conditions listed in the table.

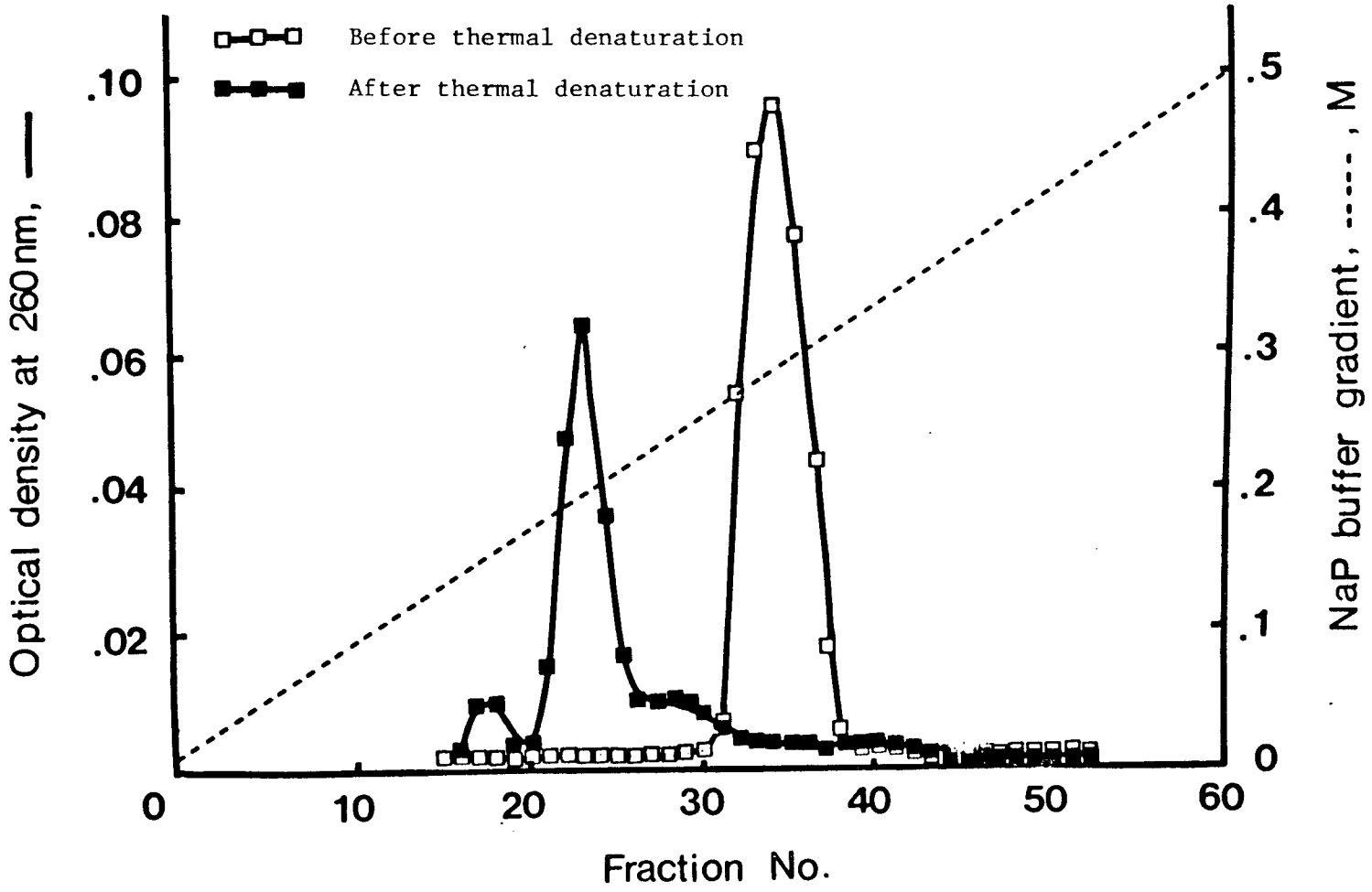


Figure 29 : HAP chromatography of DNA isolated from HC-, TA- or TA-UV treated mouse L-929 fibroblasts

(b) Denaturation-renaturation kinetics

Thermal scanning analysis of DNA has been used convincingly by Pathak et al. (1977) to demonstrate cross-linking of skin DNA by 8-MOP after exposure to UV-A irradiation. With this technique, the cross-linked DNA is detectable by virtue of its characteristic denaturation-renaturation profile (Figure 30). Investigation for cross-linking of DNA from glucocorticoid treated and glucocorticoid plus UV-A treated fibroblasts were made using this technique with a positive control of DNA isolated from 8-MOP treated and UV-A irradiated mouse L-929 fibroblasts. The results of this study are summarized with the aid of the optical melting curves and renaturation profiles shown in Figures 31-33.

The denaturation profiles of Figures 30-33 demonstrated no appreciable difference in either T_m or hyperchromicity between DNA samples of untreated fibroblasts (Figure 33) and those of fibroblasts with various treatments (Figures 30-32). However, the DNA isolated from 8-MOP and UV-A treated fibroblasts, when cooled to 50°C after thermal denaturation up to 90°C, showed clear evidence of renaturation reflected in the renaturation profile (Figure 30). The percent renaturation of cross-linked DNA was 77.7 ± 6.0% (mean ± S.E. n = 5). On the other hand, DNA isolated from (i) untreated L-929 and (ii) UV-irradiated L-929, as well as the calf thymus DNA standard gave only 9.5 ± 1.8% (mean ± S.E., n = 4 each) of renaturation under the same cooling conditions (Figures 33 and 31 respectively).

The DNA samples isolated from hydrocortisone- or triamcinolone acetonide-treated, or triamcinolone acetonide - UV treated human or mouse L-929 fibroblasts consistently had less than 10% (8.1 ± 1.1%, n = 6) of renaturation as the denatured samples were cooled to 50°C, demonstrating no detectable cross-linking due to the treatment of glucocorticoid or

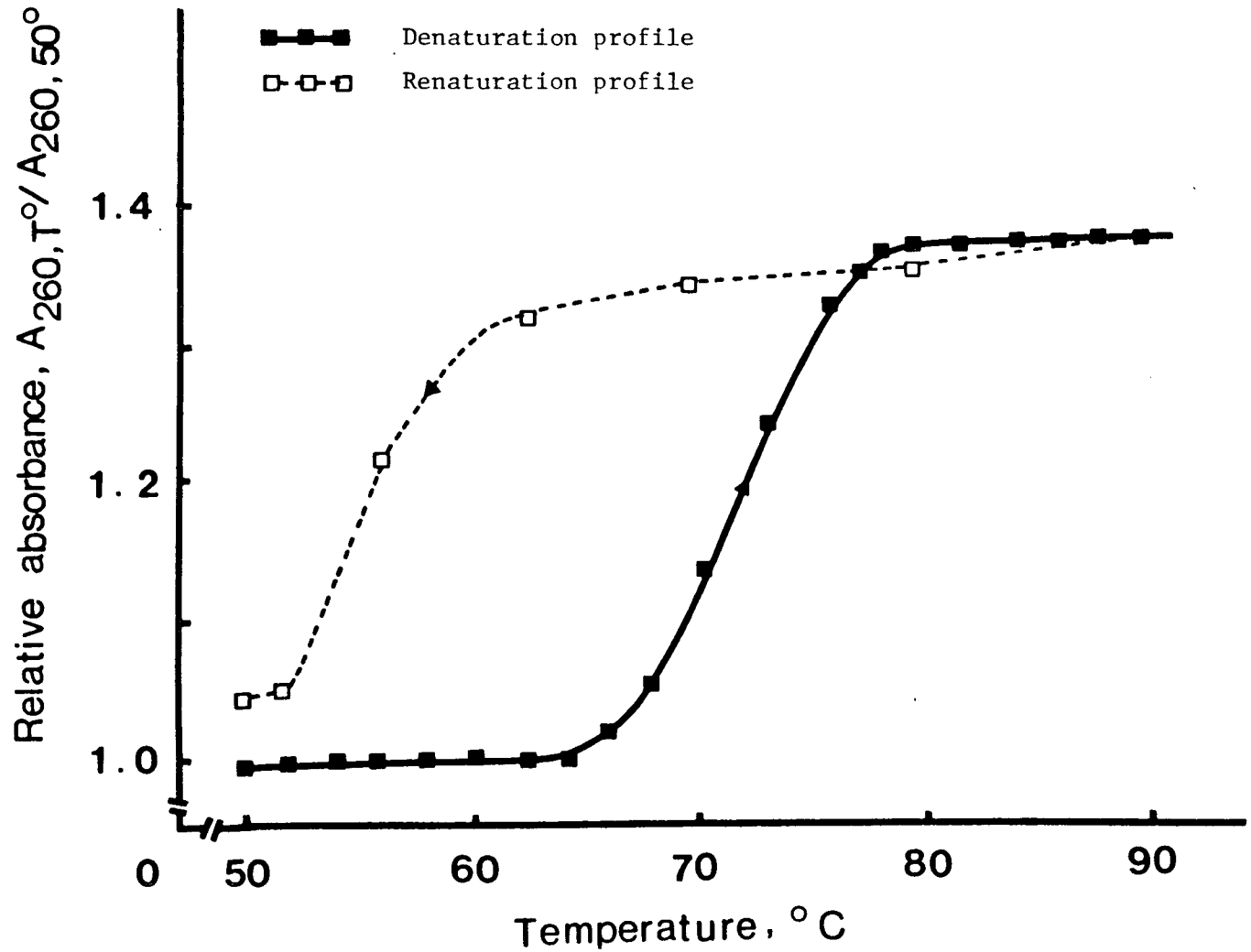


Figure 30 : Thermal scanning analysis of DNA isolated from (8-MOP)-treated and UV-A irradiated mouse L-929 fibroblasts

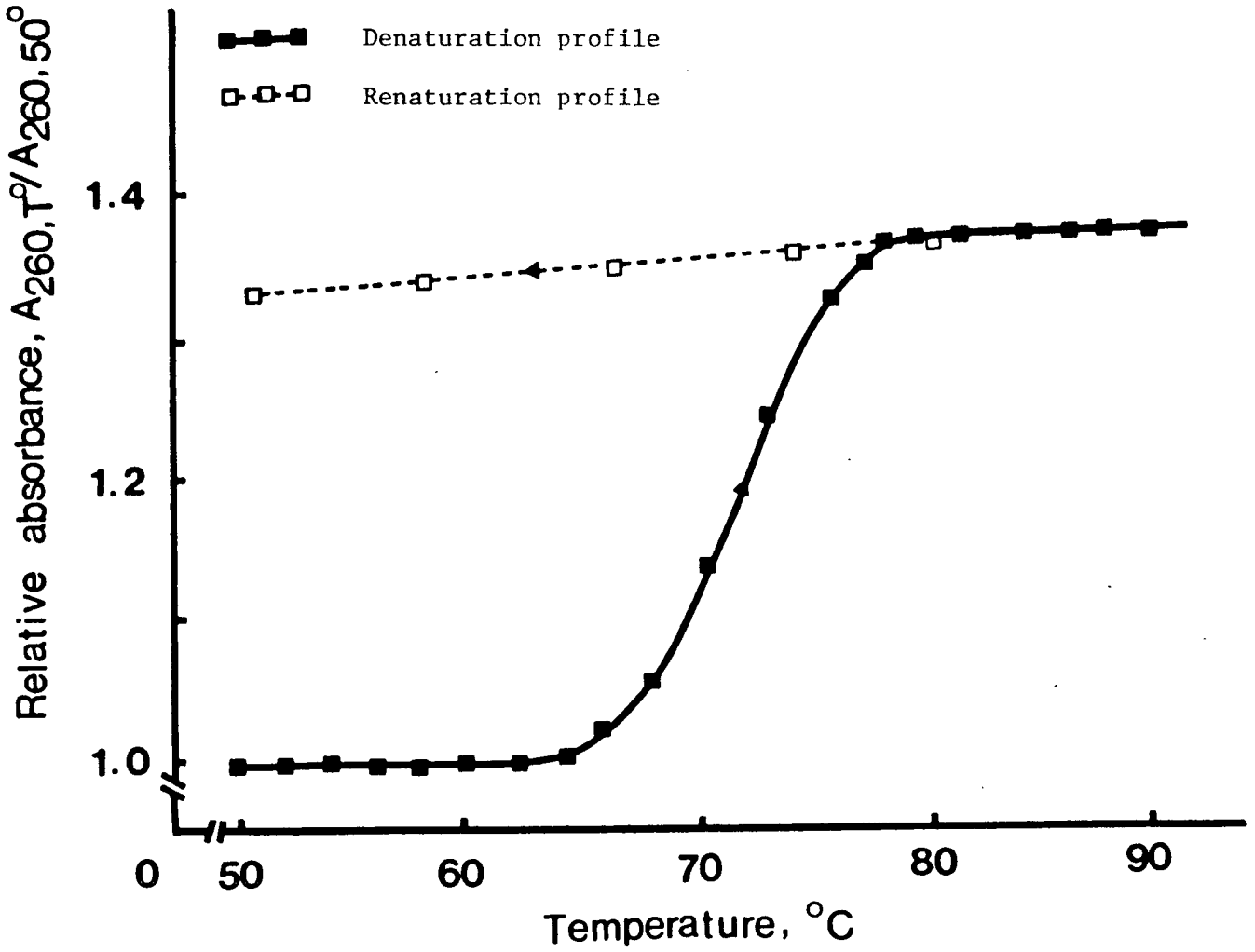


Figure 31 : Thermal scanning analysis of DNA isolated from UV-A irradiated mouse L-929 fibroblasts

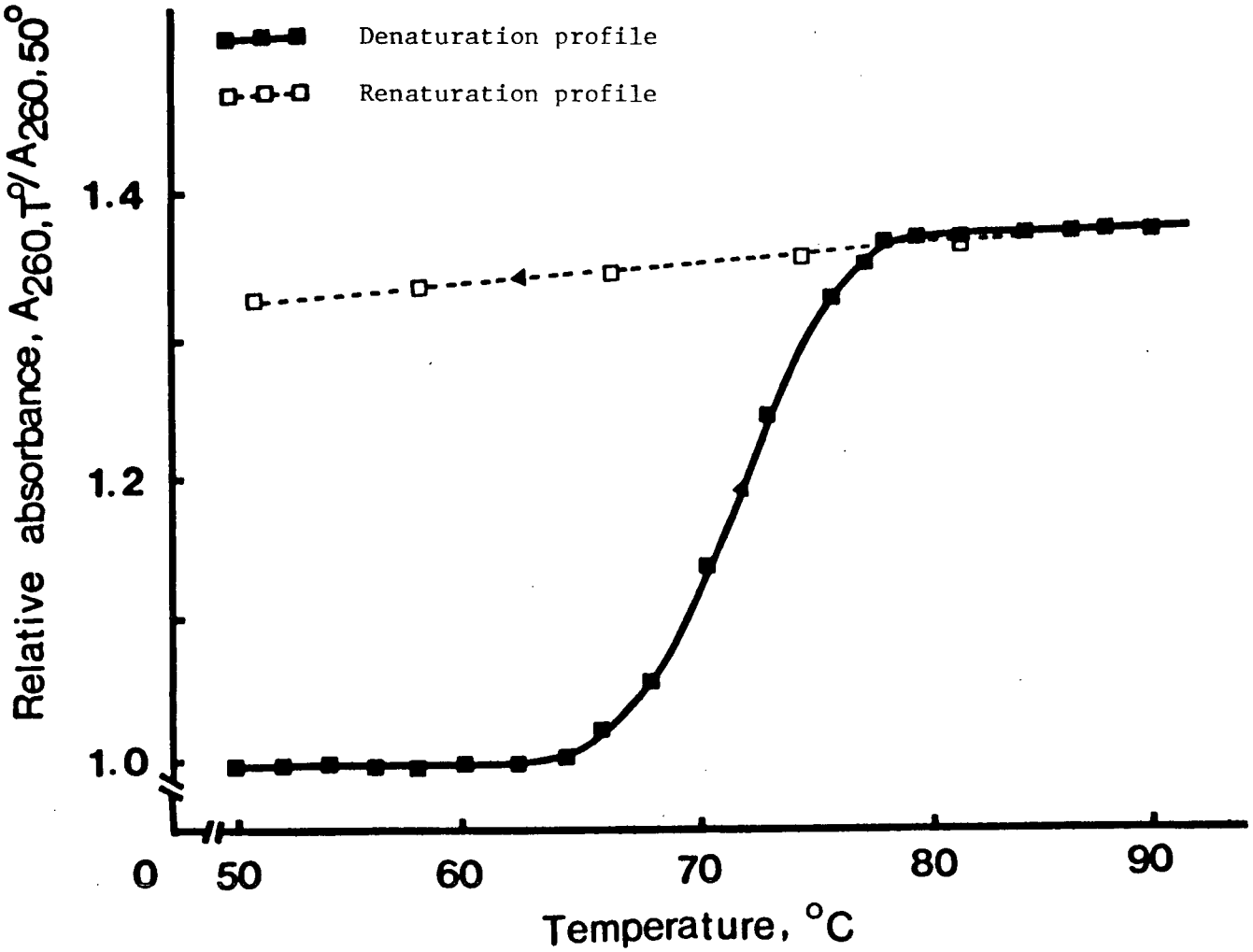


Figure 32 : Thermal scanning analysis of DNA isolated from HC-, TA- or (TA-UV)-treated mouse L-929 fibroblasts

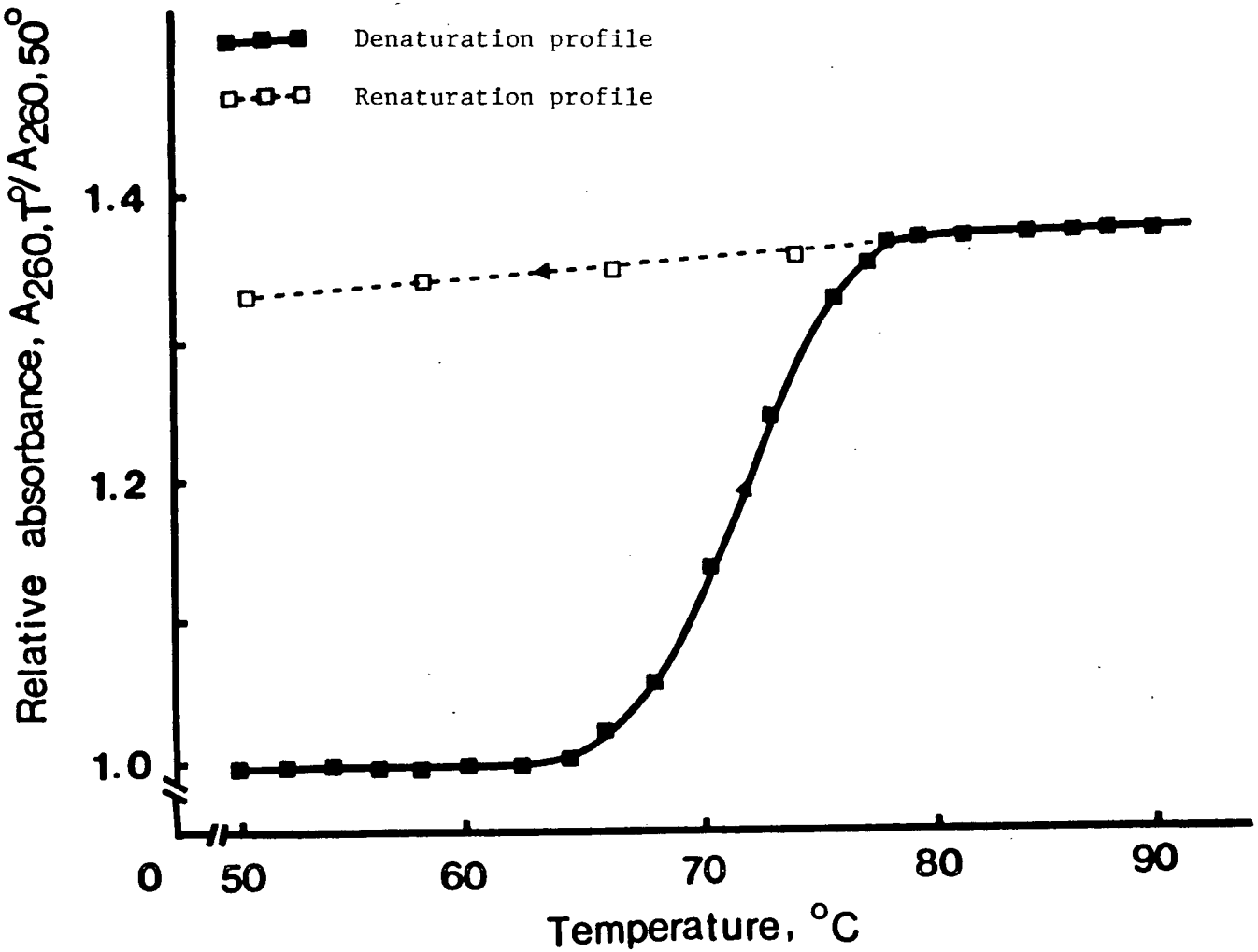


Figure 33 : Thermal scanning analysis of DNA isolated from untreated mouse L-929 fibroblasts

to the combination of triamcinolone acetonide treatment and UV-A irradiation (Figure 32).

The approximate limit of the detectability of cross-links by the optical renaturation method was 4% (Cech et al., 1979).

Based on the data of both HAP chromatography and denaturation-renaturation kinetics, neither the treatments with hydrocortisone or triamcinolone acetonide nor the combination of triamcinolone acetonide and UV-A irradiation caused detectable cross-linking of DNA in cultured human and mouse L-929 dermal fibroblasts under the experimental conditions.

(C) Retention of ^{3}H-triamcinolone acetonide during the isolation of DNA

When the deproteinized cell homogenates were chromatographed through HAP columns, only background levels of radioactivity could be recovered in the eluates (Table XIV). It was concluded, therefore, that deproteinization of ^{3}H-TA containing nuclei by triple extraction with $CHCl_3$/BuOH removed quantitatively all traces of radioactivity (Table XV). This observation ruled out the possibility of recovering DNA bound-glucocorticoid from whole cells by the use of this rather drastic isolation procedure. It does not exclude the possibility of a weak hydrogen bonding between the complex and the DNA as suggested by Duax et al. (1976) for deoxycorticosterone and adenine.

(D) Specific retention of TA in whole nuclei of mouse L-929 fibroblasts

(1) Description of the preparation of nuclei

The clean preparations of the nuclei were reproducibly obtained as judged by examination under the phase-contrast microscope. The nuclei were grey, oval particles with black nucleoli and regular membrane sur-

Table XIV HAP chromatography of deproteinized DNA from ^{3}H-TA treated mouse L-929 fibroblasts

Fraction No.	NaP (M)	Appearance (3.2 mL in 10 mL Aquasol-2)	BKG[(a)] Count (cpm)	Recovery % (b)	Recovered counts due to ^{3}H-TA, cpm	
					Gross	Net
1	0.01	Gel	21.5	66.9	32.3	10.7
3	0.35		24.5	67.0	30.4	5.9
5	0.05		25.8	70.8	29.0	3.2
7	0.065		25.7	69.2	28.9	3.2
9	0.085		25.6	67.0	33.2	7.6
11	0.1		25.5	67.3	27.5	2.0
13	0.115	Gel with liquid	26.7	67.0	32.7	6.0
15	0.13		26.3	67.6	29.3	3.0
17	0.15		26.9	68.2	28.4	1.5
19	0.165		24.9	66.3	27.3	2.4
21	0.18		27.4	65.1	31.1	3.7
23	0.2	Clear liquid	22.9	68.9	29.4	6.5
25	0.215		26.2	68.3	28.9	2.7
27	0.23		29.8	70.2	30.2	0.4
29	0.245		28.4	68.4	26.5	-1.9
31	0.26		28.1	75.6	30.3	2.2
33	0.28		24.6	77.0	27.5	2.9
35	0.295		25.6	79.1	24.7	-0.9
37	0.31		25.9	80.7	26.5	0.6
39	0.325	Liquid with ppt.	27.4	80.6	27.5	0.1
41	0.345		27.3	82.7	25.6	-1.7
43	0.36		25.0	81.9	27.1	2.1
45	0.375		27.5	83.4	25.7	-1.8
47	0.39		25.1	85.3	28.8	3.7
49	0.41		25.9	81.6	28.7	2.8
51	0.425		25.1	89.2	26.8	1.7
53	0.44		27.8	87.0	24.9	-2.9
55	0.46		26.1	84.9	27.0	0.9
57	0.475		27.4	88.6	28.1	0.7
59	0.49		23.9	86.0	24.7	0.8

(a) Background counts of individual eluents

(b) Recoveries obtained with ^{3}H-toluene standards

Table XV Quantitative removal of radioactivity by deproteinization with $CHCl_3$/ BuOH mixture

$CHCl_3$/BuOH extract	Appearance (0.5 mL, 0.1 N NaOH in 10 mL Aquasol-2)	BKG[a] Count, cpm	Recovery % (b)	Recovered counts due to ^{3}H-TA, cpm		
				Gross (n = 4)	Net	(c)
First	Clear liquid	1807.8	88.2	2555.8 ± 75.9	748.0	848.1
Second	Turbid liquid	43.8	88.7	355.8 ± 11.0	312.0	351.8
Third	Turbid liquid	119.6	85.6	555.0 ± 28.9	435.4	508.6
Total						1708.5 ± 93.3

(a) Background counts of individual extracts

(b) Recoveries obtained with ^{3}H-toluene standards

(c) Adjusted for % recovery

Total radioactivity in nuclei before deproteinization was 1705 cpm; therefore, the total recovery of radioactivity due to deproteinization was 100.2 ± 5.5%

faces. Only a faint "skeleton" of cytoplasm was noticed. Most of the granular cytoplasmic contents had been solubilized.

The uniformity of the preparation was satisfactory as reflected by coefficients of variation of less than 10%, among triplicate samples of measurements of radioactivity, DNA and protein.

(2) Time profile of specific retention on whole nuclei

(a) Gross uptake per pooled cells of 5 plates

The ^{3}H-TA taken up into the nuclei was measured by solubilizing the isolated nuclei from the pooled cells of 5 plates, containing approximately 4 - 6 x 10^{6} per plate, followed by the liquid scintillation counting of aliquots of the ^{3}H-TA in solubilized nuclei. The time profile of this process of uptake was examined by harvesting cells 0.5, 6, 24, 48, 72 and 96 hours after addition of ^{3}H-TA to the cells.

The amounts of ^{3}H-TA retained by nuclei were tabulated as cpm/5 plates and fmole/5 plates in Table XVI. The nuclear retention of ^{3}H-TA increased with time from 12 to 361 fmole/5 plates reaching a maximum at 72 hr. The data were uncorrected for variations in nuclear DNA and protein content which occurred during the period of 96 hr incubation examined.

In Figure 34, the amount of ^{3}H-TA translocated into the nuclei increased markedly from 0.5 to 6 hours of incubation, approximately tenfold. The amount of ^{3}H-TA in nuclei was then maintained relatively unchanged until 48 hours, at which time, the ^{3}H-TA was further taken up to about twice the level at 24 hours. The amount of nuclear retention then leveled off again over the remaining time period examined of 48 to 96 hours.

The increase in nuclear retention of ^{3}H-TA during the first 6

Table XVI Gross uptake of ^{3}H-TA into the nuclei of pooled cells from 5 plates

Incubation time, hr.	Gross nuclear retention[a]			
	cpm/5 plates		fmole/5 plates[b]	
	A[c]	B[d]	A[c]	B[d]
0.5	366 ± 79 (20%)[e]	48 ± 49 (100%)[e]	12.1 ± 2.6	1.6 ± 1.6
6	4035 ± 1054 (26%)	182 ± 95 (52%)	131.5 ± 34.7	6.0 ± 3.1
24	3483 ± 441 (13%)	235 ± 49 (21%)	114.8 ± 14.5	7.7 ± 1.6
48	7861 ± 1824 (23%)	341 ± 174 (51%)	259.1 ± 60.1	11.2 ± 5.7
72	10962 ± 2451 (22%)	907 ± 310 (34%)	361.3 ± 80.8	29.9 ± 10.2
96	7519 ± 1819 (24%)	707 ± 564 (80%)	247.8 ± 60.0	23.3 ± 18.6

(a) n = 6, average of six measurements from two independent series of experiments

(b) TA : 3.296×10^{-2} fmole/cpm

(c) 10^{-8} M ^{3}H-TA without unlabeled TA

(d) 10^{-8} M ^{3}H-TA with unlabeled TA

(e) C.V. (coefficient of variation) =(S.D./mean)x 100%

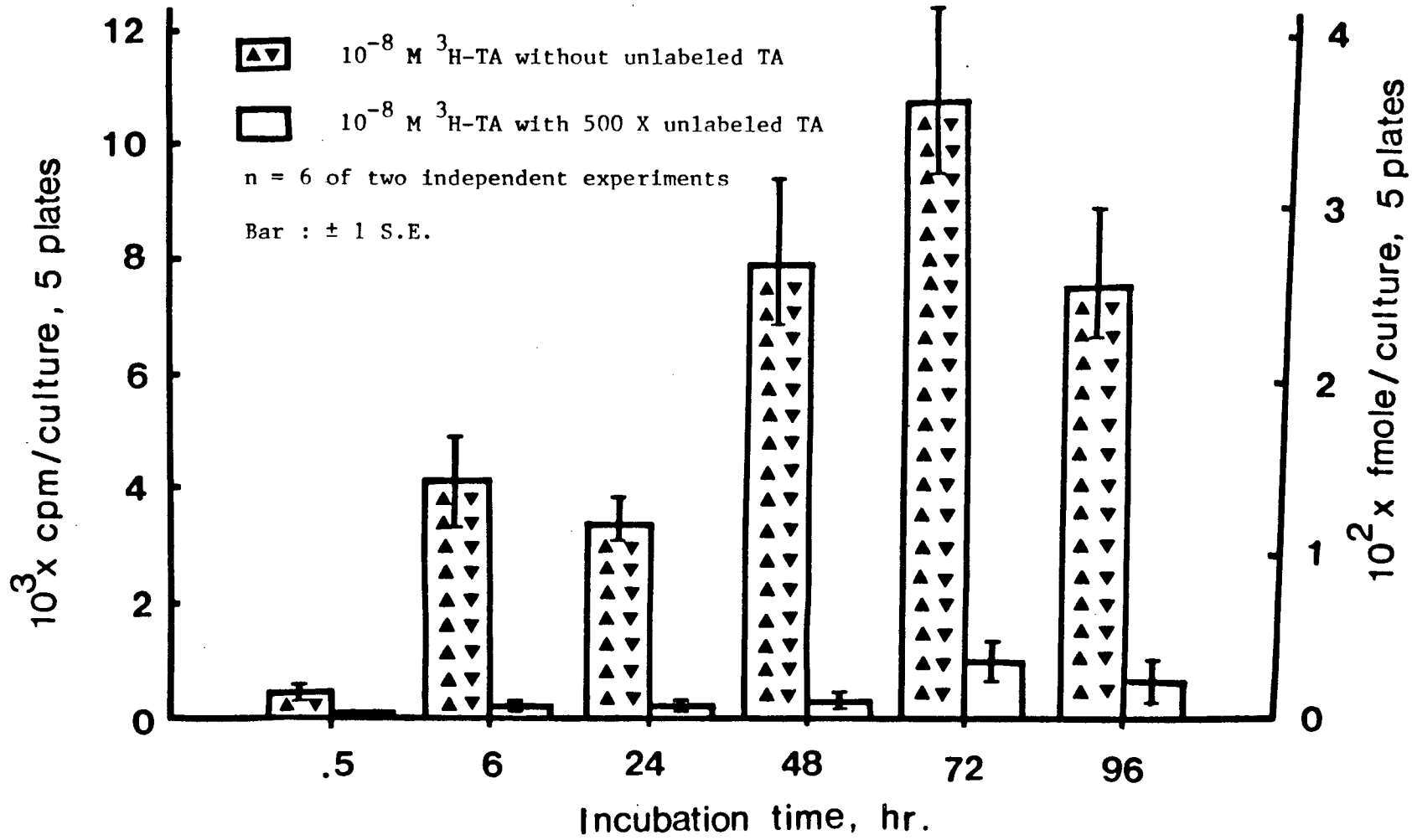


Figure 34 : Gross uptake of ^{3}H-TA into whole nuclei of pooled cells of 5 plates.

hours of incubation was gradual since the DNA content and the number of cells did not change appreciably during this period.

(b) Specific nuclear retention of ^{3}H-TA normalized for equal amount of DNA or nuclear protein

The data presented above were normalized on the basis of DNA or nuclear proteins since both new DNA and proteins were synthesized during the incubation periods up to 96 hr. The normalized data are presented in Figures 35 and 36.

The ^{3}H-TA specifically retained in nuclei, expressed as fmole/µg DNA, ranged from 0.762 ± 0.180 (mean ± S.E., n = 9 of three independent experiments) to 2.775 ± 0.645 (n = 6) fmole/µg DNA varying with the periods of incubation time (Table XVII). These data were comparable to literature values for other lines of cells: for dexamethasone, it was 2.3 fmole/µg DNA in GC cells and 1.5 fmole/µg DNA in S49 cells after 4 hours of incubation (Johnson et al., 1979), and 1.29 ± 0.2 (mean ± S.E., n = 17) pmole/mg DNA in rat hepatoma cells after at least 30 minute incubation (Rousseau et al., 1973).

The time profile of this specific nuclear retention of ^{3}H-TA is illustrated in Figure 35. As can be seen, a marked increase in nuclear retention occurred between 0.5 and 6 hours, then a significant drop was observed at 24 hours. The specifically retained TA levels in nuclei remained constant afterwards up to 96 hours (Figure 35).

A similar profile of specific nuclear retention was observed when data were normalized for equal amounts of nuclear proteins (Figure 36). The maximal retention was achieved by 6 hours with a significant drop to a plateau level afterwards which remained at constant level up to the 96 hours measured.

The kinetics of nuclear binding of glucocorticoids during the

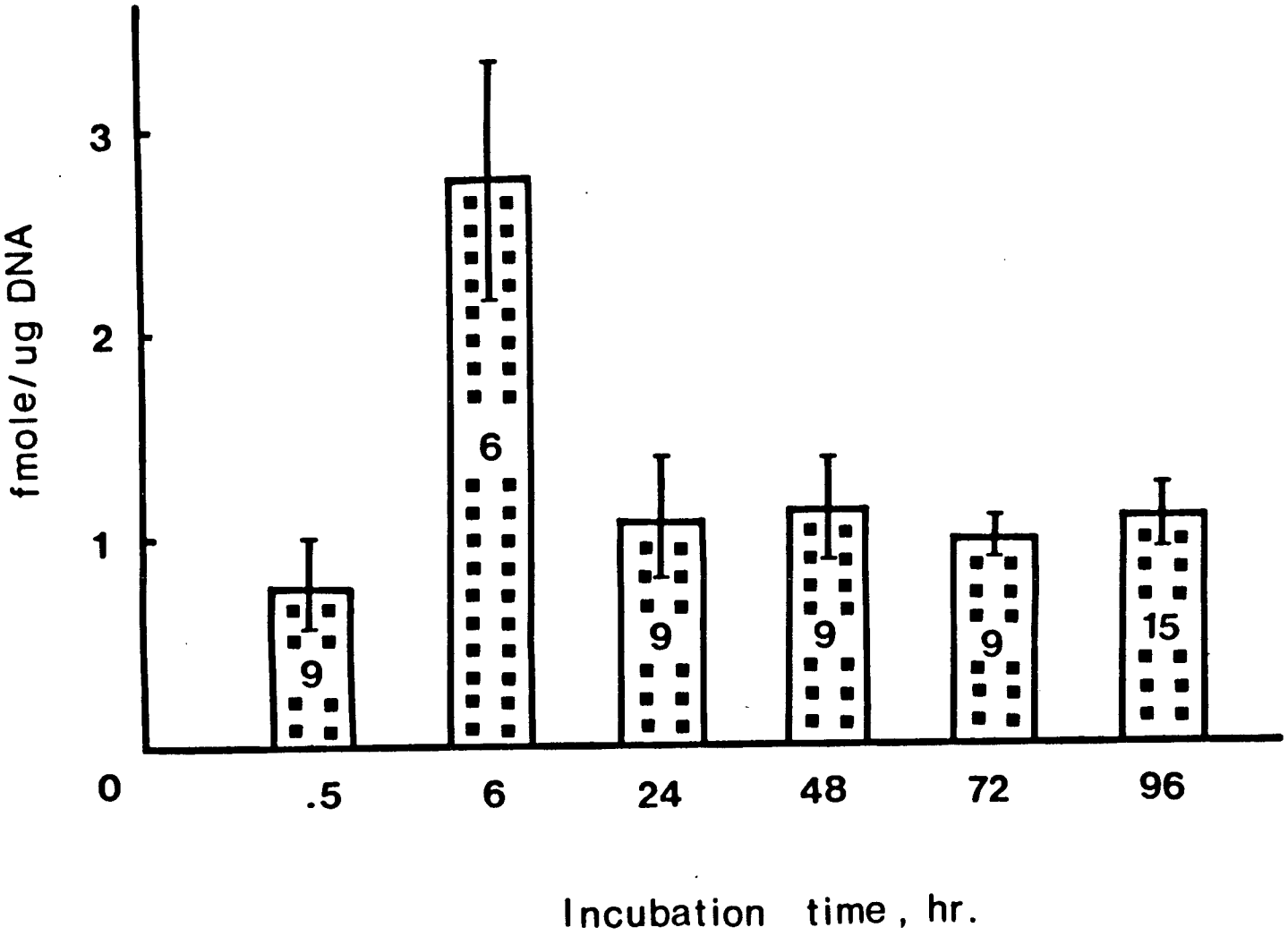


Figure 35 : Specific retention of TA in whole nuclei, normalized on DNA basis

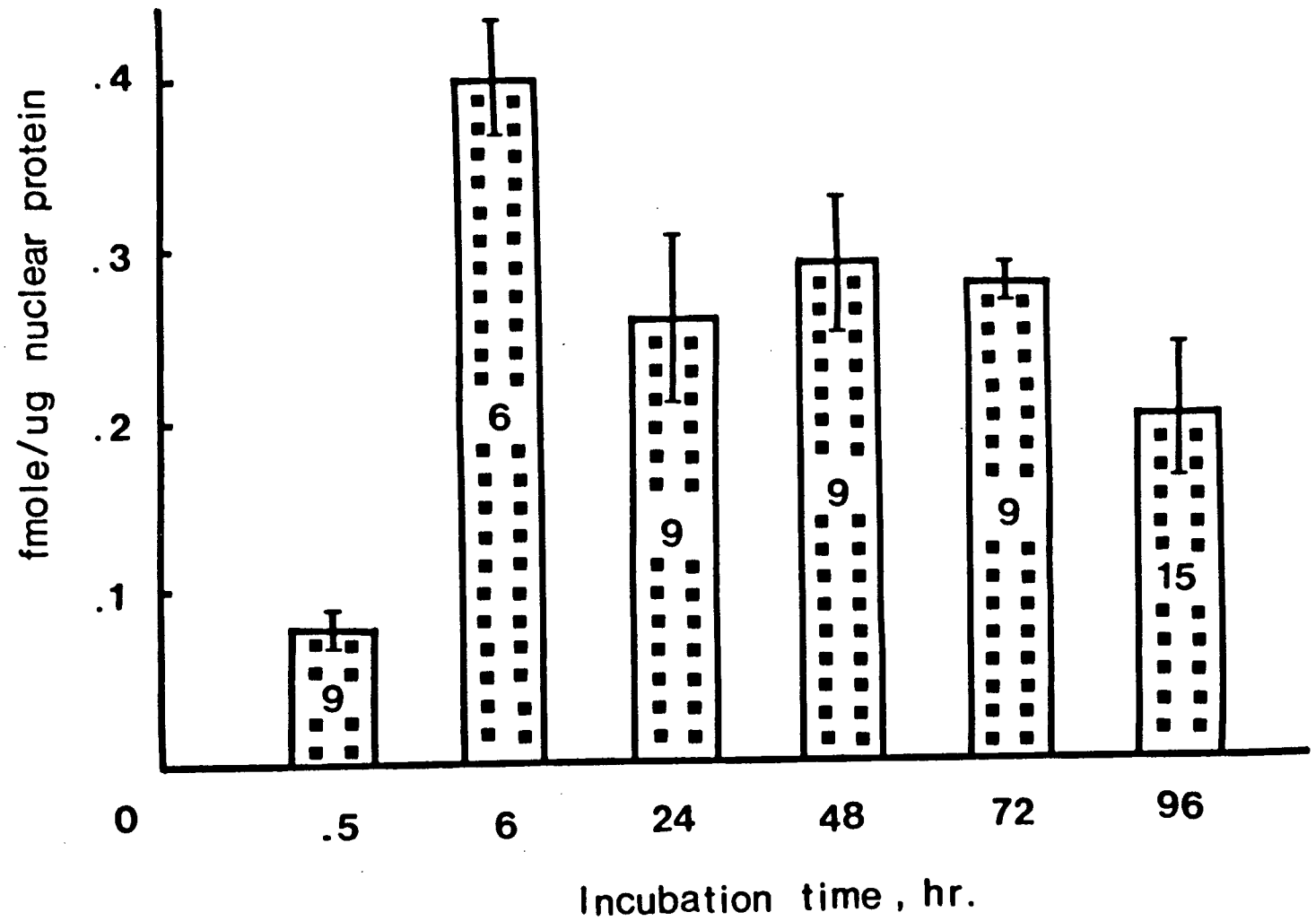


Figure 36 : Specific retention of TA in whole nuclei, normalized on nuclear protein basis

Table XVII Specific retention of TA in whole nuclei

Incubation time, hr.	No. of samples[a]	Specific retention[b]	
		fmole/µg DNA	fmole/µg nuclear proteins
0.5	9	0.762 ± 0.180	0.073 ± 0.011
6	6	2.775 ± 0.645	0.396 ± 0.042
24	9	1.076 ± 0.293	0.264 ± 0.053
48	9	1.125 ± 0.248	0.287 ± 0.041
72	9	0.998 ± 0.115	0.283 ± 0.011
96	15	1.115 ± 0.140	0.206 ± 0.042

(a) Number of samples of 2 - 5 independent experiments

(b) Mean ± S.E.

first few hours have been widely studied by various procedures (e.g. Rousseau et al., 1973; Rousseau, 1975; Ishii et al., 1972; Middlebrook et al., 1975). However, no data have been reported on the effect of prolonged incubation on the nuclear retention of TA.

Rousseau et al. (1973) reported that in hepatoma cells exposed to dexamethasone at 37°C, the nuclear binding of steroid reached a maximum within 30 minutes and then leveled off. Rousseau (1975) also claimed that the plateau persisted as long as the steroid was present. However, his study of the nuclear binding of dexamethasone lasted only 90 minutes, with only the 90 minute data point beyond 30 minutes to demonstrate the plateau (Figure 5).

Ishii et al. (1972) and Middlebrook et al. (1975) studied the subcellular distribution of ^{3}H-triamcinolone acetonide in mouse L-929 fibroblasts, and reported that the time profile of the TA-binding in the soluble cytosol fraction reached a maximum by 20 minutes and remained constant for 6 hours (Ishii et al., 1972). However, no comparable time profile for nuclear binding was reported in their studies.

There are good reasons to investigate nuclear retention beyond the 6 hours commonly studied, especially when the cells are exposed to large concentration gradients over long periods in dermatological uses of glucocorticoids.

The generation time of the L-929 fibroblasts is about 23 hours (Baserga, 1976). During this time, the susceptibility of the cells to drug action varies. New proteins and nucleic acids are synthesized. The cell divides after 23 hours. A DNA cycle with progressively increasing number of strand breaks has been suggested (Collins, 1977), posing the question whether DNA is more accessible to glucocorticoids at one time than another.

The glucocorticoids, according to some (e.g. Voorhees, 1977b), act during the G_2 phase of the cell cycle as inhibitors of mitosis. According to others, glucocorticoids act at all stages of the cell cycle (Baserga, 1976). The issue is unsettled. More information on glucocorticoid action in nuclei is needed. Clinically, the prolonged exposure of glucocorticoids is of interest, since both the development of resistance to glucocorticoid action (DuVivier, 1976; DuVivier and Stoughton, 1977) and adverse effects require a long time to develop.

Another aspect of the previous studies of nuclear retention of glucocorticoids, which may introduce uncertainty, is that the published data have been obtained by means of measurement of crude homogenates. Rousseau et al. (1973), for instance, separated the cell homogenate of ^{3}H-dexamethasone-incubated hepatoma cells by centrifugation at 1,200 x g for 5 minutes into cytosol-containing supernatant and a pellet. After washing, the radioactivity of the pellet was measured. Similarly, Ishii et al. (1972) and Middlebrook et al. (1975) fractionated the ruptured cells by centrifugation at 7,000 x g for 3 minutes into cytosol-containing supernatant and the nuclear pellet. In contrast, the measurements reported here involved the isolation of clean intact nuclei, rather a crude fraction of a nuclear pellet, prior to the measurement of radioactivity. The data obtained here had much less possibility of being distorted by contamination with cytosol radioactivity.

This study clearly demonstrated the presence of ^{3}H-TA in the nuclei of cultured fibroblasts throughout the time period of 96 hr examined. Therefore, the absence of cross-linking of DNA with glucocorticoids reported in Results and Discussion section B could not be ascribed to an absence of glucocorticoids in the nuclei of the fibroblasts examined for cross-linking.

(3) Effects of TA on total nuclear protein and DNA content in nuclei

(a) Selection and reliability of protein and DNA assays

Bio-Rad protein assay kit was employed to quantitate the contaminating protein of purified DNA, and the protein contents of preparations of nuclei or chromatin. Commercially obtained calf thymus DNA which is widely accepted as substantially protein-free preparation served as a control.

This assay has advantages over other protein assays, especially the widely used Lowry method (Lowry et al., 1951), because (i) it is much easier to use, requiring one reagent and five minutes to perform as compared to three reagents and 30-40 minutes typical for the Lowry assay, (ii) the absorbance of dye-protein complex is relatively stable, not requiring the critical timing necessary for the Lowry assay and (iii) it is free from most of the interferences which limit the application of the Lowry assay. The sensitivity limit of the assay is 1 μg per sample, comparable to that of Lowry method.

The reproducibility of the calibration curve was satisfactory. The C.V. of the slopes of the curves was less than 5% among runs.

The reagent blank containing the sample buffer and the dye reagent resulted in a reddish-brown solution with an absorbance characteristically about 0.425. The relatively high color yield of the blank was normal (Bio-Rad bulletin, 1979), and did not affect the linearity, reproducibility or sensitivity of the assay.

DNA interferes with the assay, however, when present in concentrations of more than 1 mg/mL (Bio-Rad bulletin, 1979). The highest DNA content in the samples of this project was kept to less than 0.2 mg/mL; therefore, no interference by DNA was noted.

The solubilizing buffer used does not interfere with the Bio-Rad

assay, although all five of its components, Tris-HCl, $CaCl_2$, $MgCl_2$, dithiothreitol and glycerol are known to interfere with the Lowry assay.

The widely used Burton's technique for DNA determinations gave satisfactory reproducibility. Maximum difference in slopes of calibration curves was 3% (C.V.). It was found however that our use of dithiothreitol-containing buffer for solubilizing nucleus required prior dialysis against 0.5 N $HClO_4$, otherwise, no color was developed in the presence of the solubilizing buffer. Apparently, the reducing agent, dithiothreitol, prevented the formation of Schiff's base between the amine and deoxyribose. The dialysis removed the interference completely.

The technique is specific for nuclear DNA. RNA, in amounts of 2000 µg gives a detectable color equivalent to 1.4 µg of DNA (Burton, 1956). Considering the minute quantities of RNA present in nuclei, the extent of this interference was considered negligible. There was no interference due to the presence of protein in test samples.

(b) Variations in the nuclear protein level of TA-treated and TA-free mouse L-929 fibroblasts as a function of time

The total nuclear protein level in pooled cells of 5 plates were determined using triplicate aliquots of solubilized nuclei by Bio-Rad protein assay.

In Figure 37, the total nuclear protein contents, in mg, were plotted against the incubation time, 0.5 - 96 hours. Control cultures, without TA treatment, possessed relatively constant levels up to 6 hours, which corresponds to the time required for the glucocorticoid to reach its maximal nuclear retention. The nuclear protein levels approximately doubled, first, by 24 hours, and then again by 48 hours. Beyond 48 hours, the nuclear protein contents no longer increased and leveled off up to 96 hours (Figure 37).

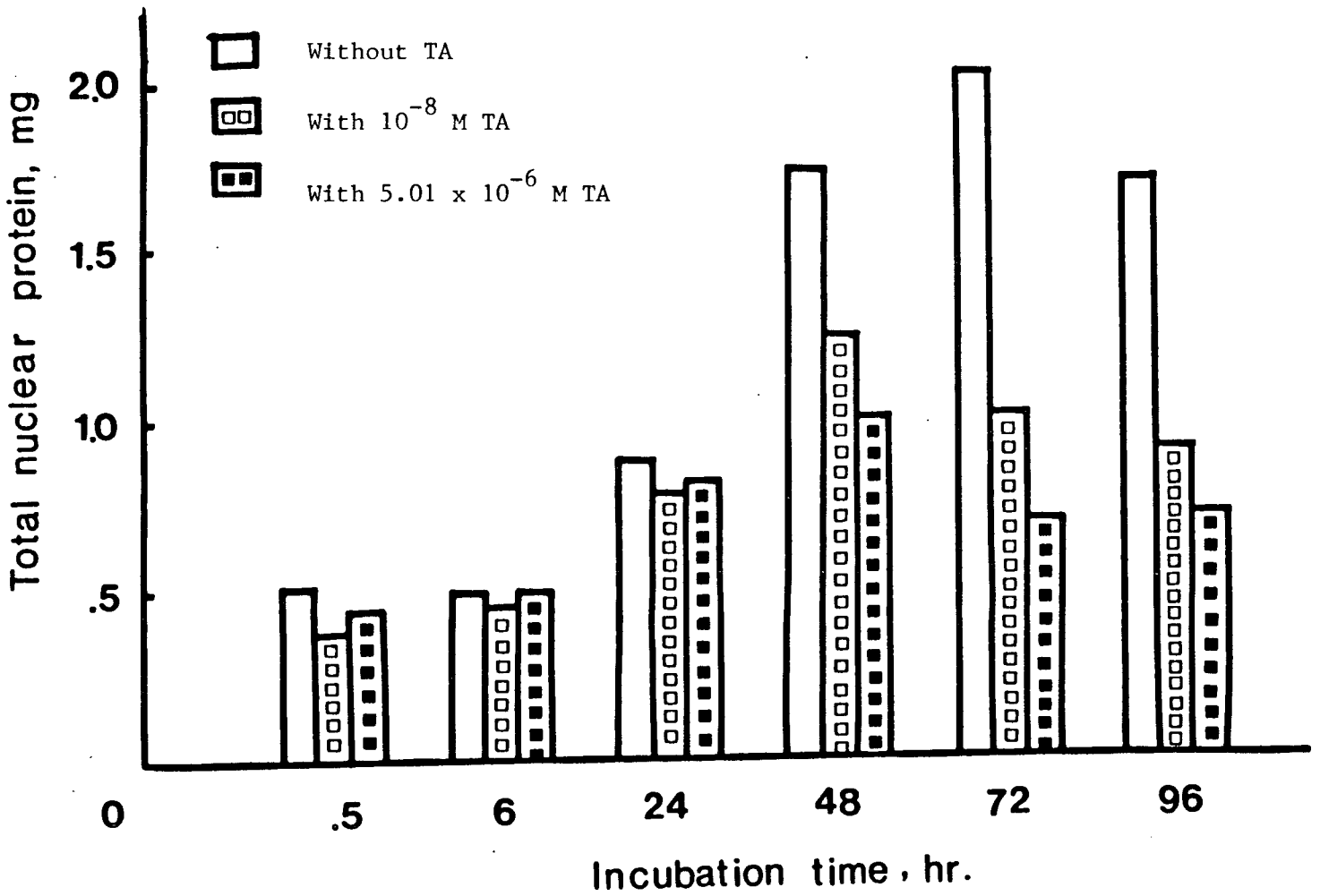


Figure 37 : Total nuclear protein levels in TA-free and TA-treated cultures as a function of time. Maximum C.V. is 5%, n = 3.

TA-treated cultures at two concentrations of 10^{-8} M and 5.01 x 10^{-6} M showed profoundly lower nuclear protein contents (Table XVIII). Plotted as percent suppression relative to control culture protein levels against time (Figure 38), it is seen that the inhibition of protein formation becomes more pronounced at the later stages of incubation, 24 to 96 hr. The nuclear protein levels were nearly 50% lower than in untreated cultures after 24 to 96 hr of TA incubation.

Pratt in his review (1978) stated that general cellular protein synthesis in L cells was not inhibited by glucocorticoids, but that selective inhibition to the synthesis of some individual proteins might occur. Wong and Aronow (1976) reported selective inhibition of incorporation of radioactive amino acids into a lysine-rich histone component in L cell nuclei. No further studies of TA effect on nuclear protein levels for various incubation times seem to have been reported. The higher dose of TA, 5.01 x 10^{-6} M exerted appreciably greater effect than that of 10^{-8} M only after 48 hours of incubation.

(c) Variations in the DNA content in nuclei of TA-treated and TA-free mouse L-929 fibroblasts as a function of time

The DNA content in pooled cells of 5 plates were assayed with triplicate aliquots of solubilized nuclei by Burton's diphenylamine technique.

In Figure 39, the total DNA contents, in mg, were plotted against incubation time. Control cultures without the treatment of TA maintained relatively constant levels of DNA up to 6 hours. The amount of DNA increased more than two-fold by 24 hours, and about four-fold by 48 hours. The DNA levels remained relatively constant beyond 48 hours up to 96 hours (Figure 39).

The DNA content of TA-treated cultures showed, with respect to

Table XVIII Total nuclear protein levels in TA-free and TA-treated cultured mouse L-929 fibroblasts at various incubation intervals

Incubation time, hr.	Nuclear protein content[a]				
	TA-free control	1.0×10^{-8} M TA		5.0×10^{-6} M TA	
	µg	µg	% suppression[b]	µg	% suppression[b]
0.5	505.8	378.5	25.2	481.0	5.1
6	508.3	477.7	6.0	500.1	1.6
24	942.2	801.7	14.9	821.5	12.8
48	1804.4	1308.6	27.5	1060.7	41.2
72	2008.4	1037.3	48.4	719.1	64.2
96	1702.5	917.4	46.1	731.5	57.0

(a) average of triplicate measurements. Maximum C.V. is 5%

(b) calculated as $\frac{(\text{nuclear protein})_{\text{TA-free}} - (\text{nuclear protein})_{\text{TA-treated}}}{(\text{nuclear protein})_{\text{TA-free}}} \times 100\%$

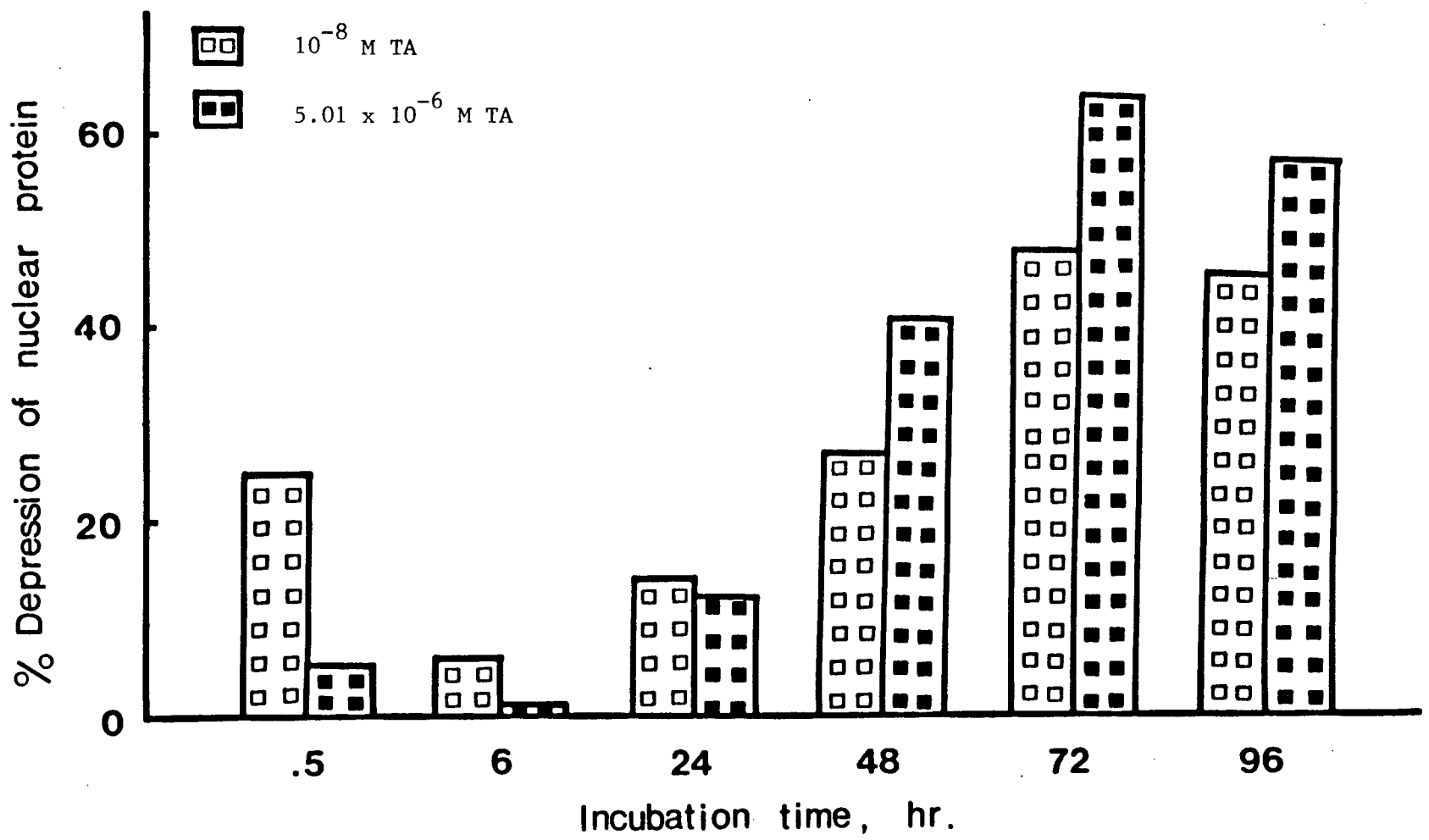


Figure 38 : Suppression of total nuclear proteins induced by TA at levels of 10^{-8} and 5.01 x 10^{-6} M

Maximum C.V. is 5%, n = 3

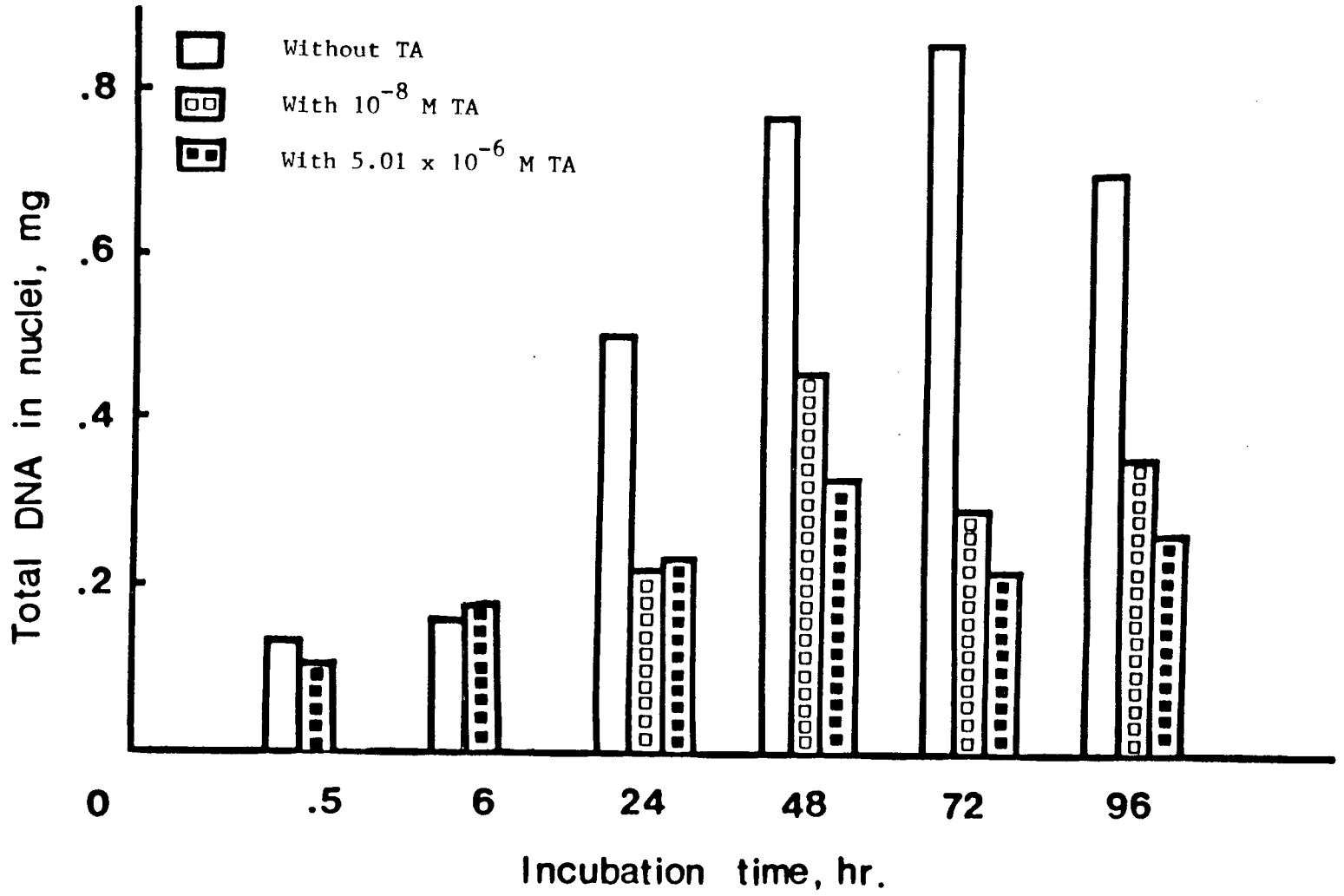


Figure 39 : DNA levels in nuclei of TA-free and TA-treated mouse L-929 fibroblasts as a function of time. Maximum C.V. is 5%, n = 3

the above controls, suppression of DNA. The suppression was similar to the TA effect on nuclear proteins, but occurred earlier, after 24 hours rather than 48 hours. The percent of suppression of DNA content varied with the concentration of the glucocorticoid, and was 40-65% for 10^{-8} M and 50-73% for 5.01×10^{-6} M TA under the same incubation conditions (Table XIX, Figure 40). At 24 hours, both concentrations of TA exerted similar degrees of suppression of DNA levels. At 48-96 hours, cultures receiving 5.01×10^{-6} M TA had a greater effect.

These inhibitory effects were expected from known glucocorticoid actions. The suppressive effect on DNA synthesis has been evaluated by measuring decreased incorporation of ^{3}H-thymidine into DNA (Pratt, 1978; Pratt and Aronow, 1966; Armelin and Armelin, 1978; Adolf and Swetly, 1979). Pratt and Aronow studied the inhibition of DNA synthesis in L-929 fibroblasts by fluocinolone acetonide, 5×10^{-7} M, and by hydrocortisone, 5×10^{-6} M at 3, 6, 12 and 24 hours. They found that the inhibition was initially evident within 6 hours. After 24 hours of exposure, the incorporation of thymidine was inhibited by about 50%. Similar studies on L-929 fibroblasts over several days of exposure to hydrocortisone demonstrated that the maximum inhibition of thymidine incorporation was about 70% and was attained at 24 hours (Seifert and Hilz, 1966). Armelin and Armelin (1978) reported that this inhibitory action of hydrocortisone, at the physiological level of 0.3 µg/mL in ST 1 cells, increased progressively with time of incubation, reaching a maximum after 20 hours. In Adolf and Swetly's studies (1979), it was observed that after 48 hours of incubation of human lymphoid cells with 10 µM triamcinolone, the inhibition of DNA synthesis was 86 ± 10%, and this effect was dose-dependent.

The data from this study show the same magnitude of suppression

Table XIX DNA levels in nuclei of TA-free and TA-treated cultured mouse L-929 fibroblasts at various incubation intervals

Incubation time, hr.	DNA content[a]				
	TA-free control	1.0×10^{-8} M TA		5.0×10^{-6} M TA	
	µg	µg	% suppression[b]	µg	% suppression[b]
0.5	137.7	-	-	131.2	4.7
6	153.6	-	-	176.1	-
24	512.4	223.4	56.4	225.3	56.0
48	774.3	462.8	40.2	317.4	59.0
72	852.8	298.8	65	234.3	72.5
96	710.1	371.6	47.7	280.4	60.5

(a) Average of triplicate measurements. Maximum C.V. is 5%.

(b) calculated as $\frac{(DNA)_{TA\text{-}free} - (DNA)_{TA\text{-}treated}}{(DNA)_{TA\text{-}free}} \times 100\%$

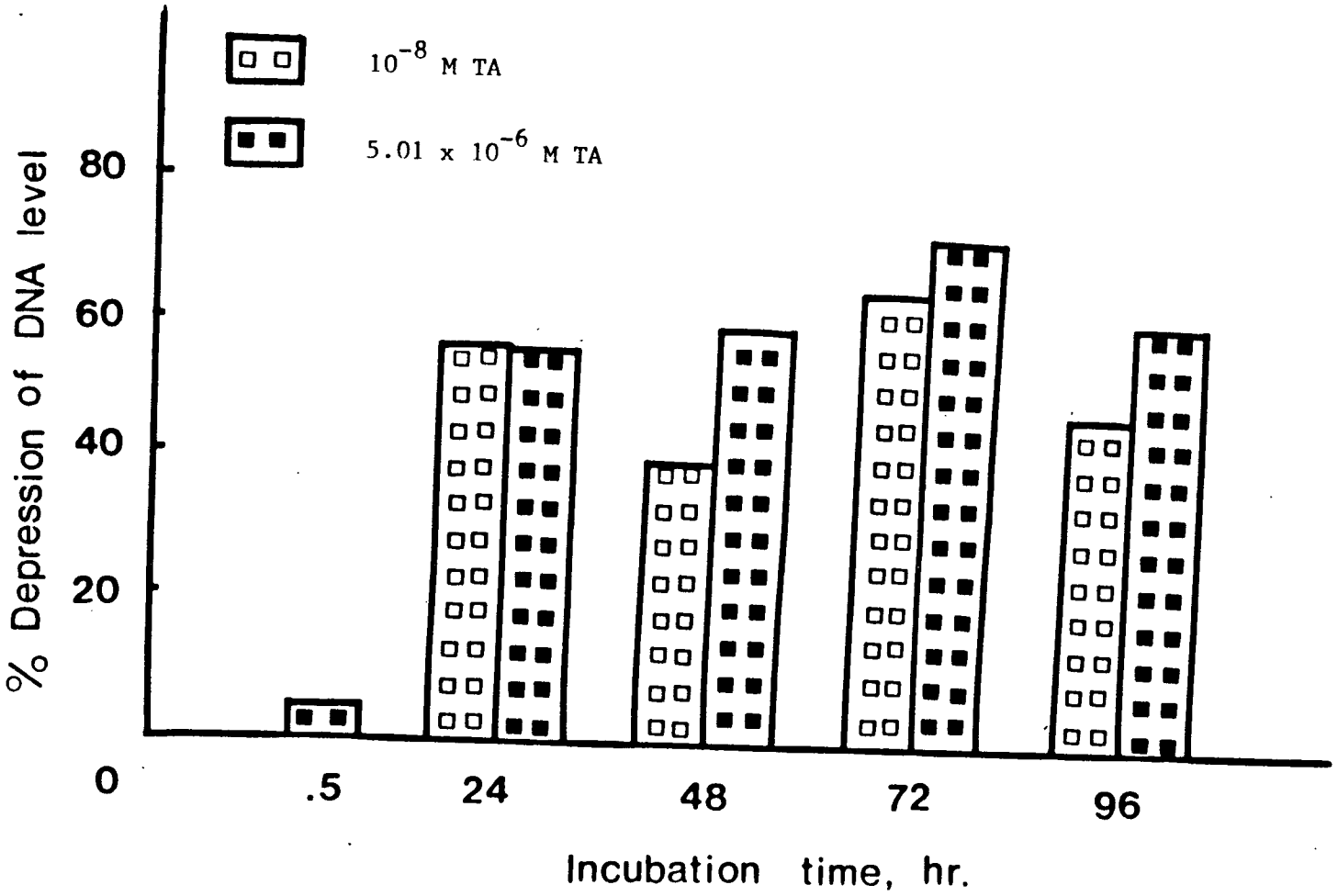


Figure 40 : Suppression of DNA level in nuclei induced by TA at concentrations of 10^{-8} M and 5.01×10^{-6} M. Maximum C.V. is 5%, n = 3

of DNA level by TA by direct measurement of the amount of DNA in nuclei as those reported in literature by indirect methods and demonstrated that although no direct effect of TA on the DNA structure (cross-linking) could be established, biological effects clearly involving DNA, and depending on the presence of TA, such as the inhibition of nuclear protein and DNA formation, do indeed occur. The data reported here confirmed that the cell line used for investigating the glucocorticoid-induced cross-linking is responsive to the treatment of glucocorticoid.

(4) Intracellular distribution of ^{3}H-TA as a function of time

The intracellular distributions of ^{3}H-TA upon the incubation with L-929 fibroblasts at 37°C for varying periods of time were determined. The harvested cells were washed three times with 5 mL of ice-cold phosphate buffered saline (PBS) to remove the adhering extracellular TA. In order to prevent the loss of intracellular TA in the washing process, unlabeled TA, 5×10^{-6} M, was added in PBS to maintain the same concentration gradient between the inside and the outside of the cytoplasmic membrane. The washed cells were lysed with 0.5% of Triton X-100 in EDTA/saline followed by one wash with ice-cold PBS. The radioactivities present in the Triton X-100 lysate and in the subsequent PBS wash were defined as the cytoplasmic TA. The nuclear TA was measured as the radioactivity in the preparation of the lysed nuclei.

The data on intracellular distribution were obtained from two to seven independent studies, each consisting of an average of three measurements. In the cases of incubation with 10^{-8} M ^{3}H-TA alone, the total amount of TA taken up into the cells increased proportionally between 0.5 and 4 hours, and then was constant up to 96 hours (Figure 41; Table XX). The distribution of intracellular TA between cytoplasm and nucleus was relatively constant with varied incubation times. The per-

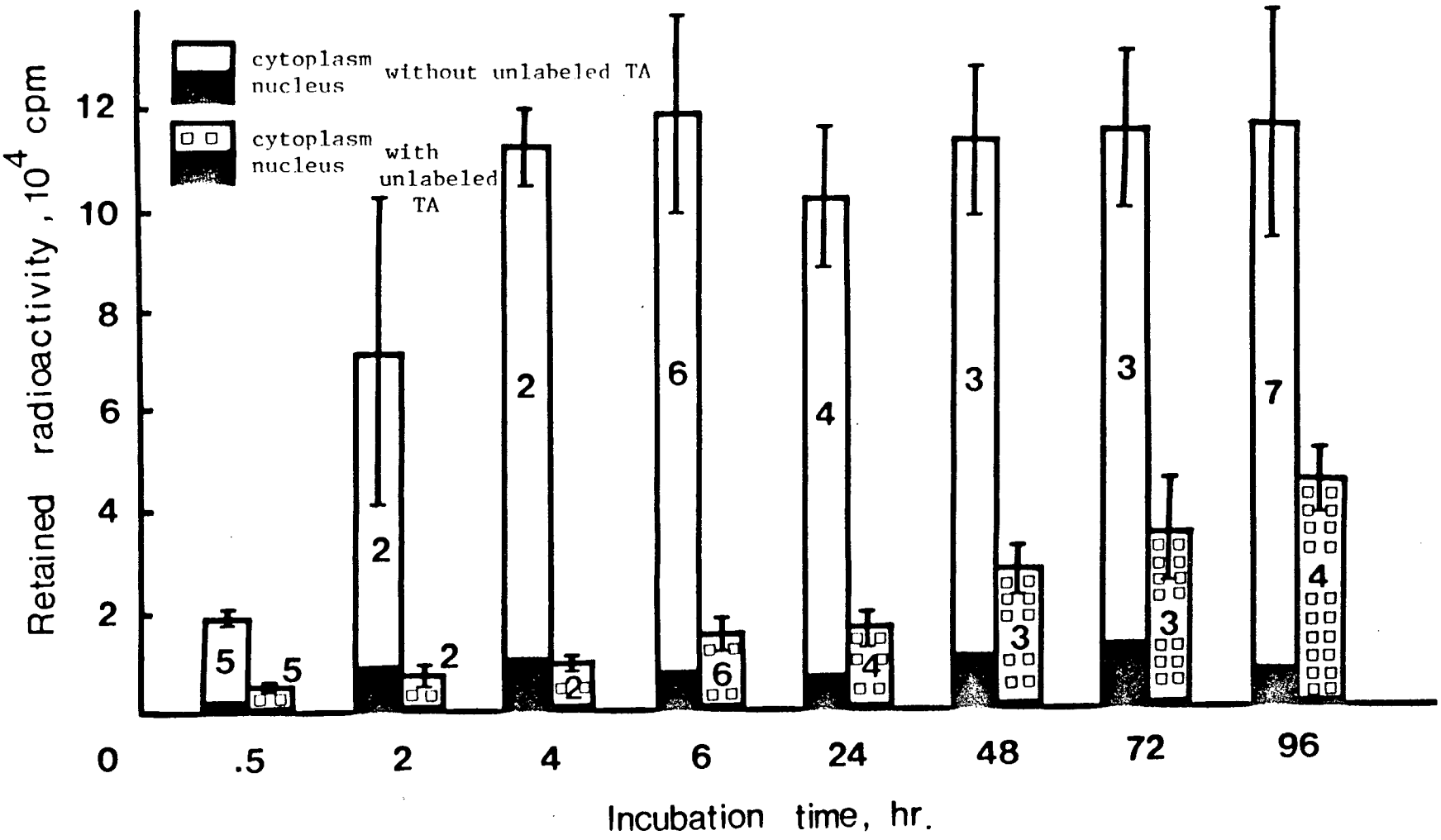


Figure 41 : Intracellular distribution of ^{3}H-TA between cytoplasm and nucleus in the absence or the presence of 500-fold unlabeled TA

Table XX Intracellular distribution of ^{3}H-TA between cytoplasm and nucleus in the absence or the presence of 500-fold unlabeled TA

Incubation time, hr.	No. of samples	Without unlabeled TA			With 500-fold unlabeled TA		
		Total intracellular activity, cpm	% distribution		Total intracellular activity, cpm	% distribution	
			Cytoplasm	Nuclei		Cytoplasm	Nuclei
0.5	15	12493 ± 1821	95.3 ± 0.9	4.7 ± 0.9	4120 ± 899	97.1 ± 0.8	2.9 ± 0.8
2	6	71088 ± 3174	88.5 ± 3.5	11.5 ± 3.5	7678 ± 1037	95.5 ± 1.8	4.5 ± 1.8
4	6	111694 ± 6761	91.0 ± 0.2	9.0 ± 0.2	9376 ± 1429	97.1 ± 0.4	3.0 ± 0.4
6	18	116396 ± 21077	94.0 ± 0.9	6.1 ± 0.9	16295 ± 4386	97.7 ± 0.5	2.4 ± 0.5
24	12	100451 ± 15906	95.0 ± 0.8	5.0 ± 0.8	17369 ± 3752	98.4 ± 0.3	1.6 ± 0.3
48	9	112491 ± 14957	90.8 ± 1.4	9.2 ± 1.4	27344 ± 5036	98.9 ± 0.5	1.1 ± 0.5
72	9	114417 ± 14563	89.4 ± 0.4	10.6 ± 0.4	39567 ± 9804	97.8 ± 0.8	2.2 ± 1.4
96	12	114835 ± 24335	93.5 ± 1.0	6.5 ± 1.0	45711 ± 6893	98.6 ± 0.2	1.4 ± 0.2

* data were presented as mean ± S.E.

centage of cellular TA in nuclei were in the range of 4.7 to 11.5% (Figure 42). On the other hand, when 500-fold of unlabeled TA was incubated together with ^{3}H-TA, the cellular TA was mainly located in cytoplasm. Cytoplasmic TA constituted 95.5 to 98.9% of cellular TA. The ^{3}H-TA taken up into nuclei under this incubation condition (with unlabeled competitors) was at a negligible level. The total amount of ^{3}H-TA transferred into cells increased gradually upon prolonged incubations between 6 and 96 hours; however, no appreciable change was observed in their nuclear levels (Figure 41).

Middlebrook et al. (1975) determined the subcellular distribution of glucocorticoid receptors in mouse L-929 fibroblasts by incubating cells at 37^{o}C for 1 hour with 10^{-8} M ^{3}H-TA, with and without the addition of 1000-fold unlabeled glucocorticoid as competitors. They found intracellular TA-receptor complexes located in cytoplasmic, nuclear extractable and nuclear residual fractions with percentages of 80 ± 8, 11 ± 7 and 9 ± 4%, respectively. The nuclear retention of ^{3}H-TA was approximately double the value reported here. However, the design of the experiment was different in several important aspects. In their studies, the cells were ruptured with a hypotonic buffer after 1 hour of incubation with TA, followed by a bringing up the cell homogenate to an isotonic condition prior to the centrifugation at 7,000 x g. They defined the supernatant as the cytoplasmic fraction. Nuclear extractable fractions were those extracted from nuclear pellets with 0.3 M KCl-0.01 M Tris buffer, leaving the non-extractable portion as the "nuclear residual fraction". In the work of Middlebrook et al., the nuclear pellets were composed of both nuclei and cytoplasmic debris; therefore, it is not surprising that their results gave the higher radioactivity in nuclear fractions than that reported here, because of contamination due to cytoplas-

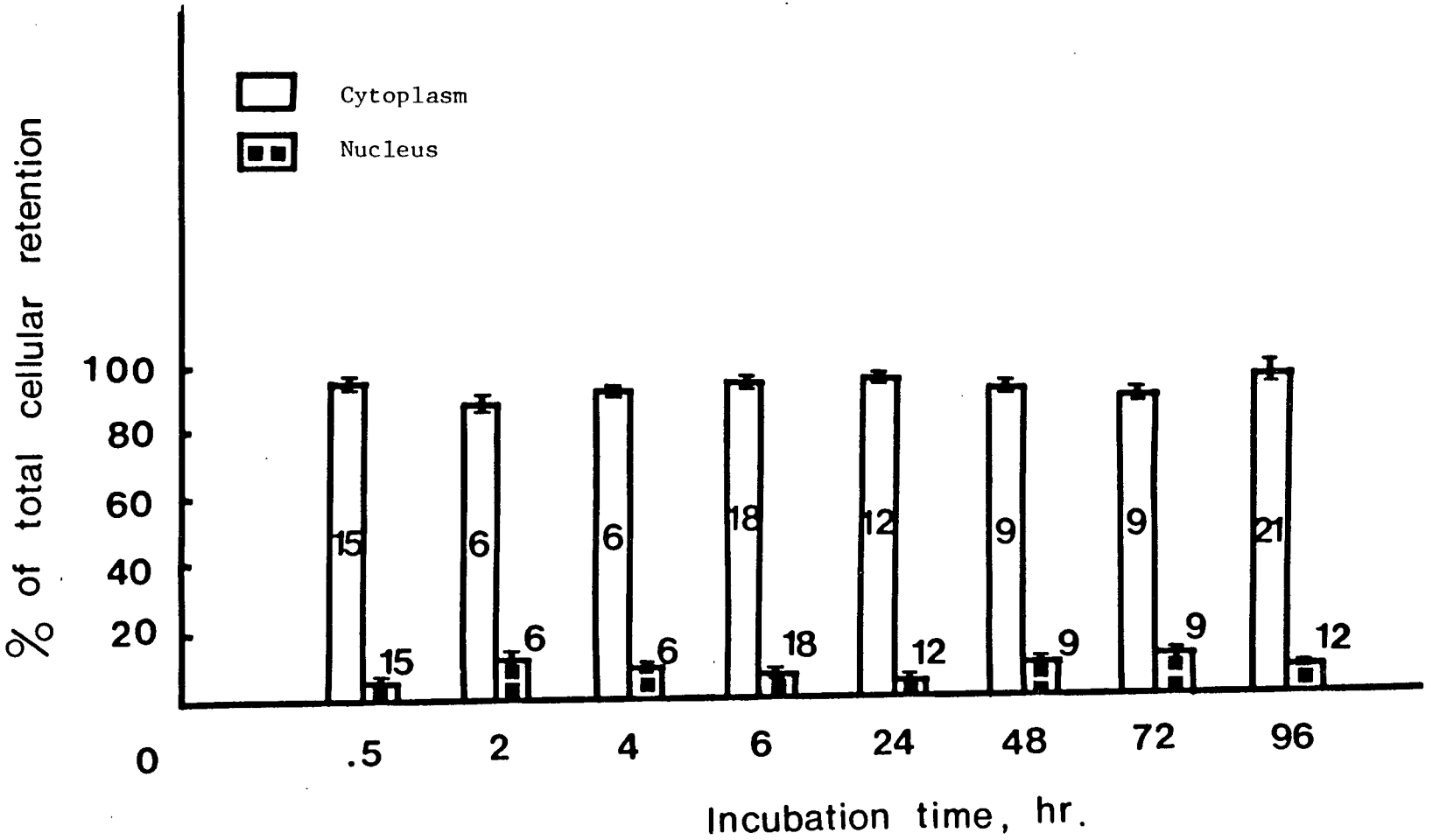


Figure 42 : Percent distribution of total cellular ^{3}H-TA between cytoplasm and nucleus

mic debris. In this thesis, the cell lysis was done more selectively by rupturing only the cytoplasmic membrane. Moreover, the nuclear retention of ^{3}H-TA was measured after isolating intact nuclei to ensure minimal contamination due to cytoplasmic particulate.

The leakage of nuclear components and TA through the nuclear membrane in the process of isolating nuclei was a possibility. As an additional precaution, unlabeled TA was included in both lysing agent and washing buffer for maintaining constant concentration gradient of TA between the inside and the outside of the nuclear membrane, which in turn, would aid in preventing net loss of ^{3}H-TA from nuclei regardless of the possible leakage of other nuclear components.

(E) Specific retention of ^{3}H-TA in chromatin

(1) Characterization of isolated chromatin

The criterion employed to characterize the isolated chromatin was the ratio of protein/DNA in chromatin. The range of this ratio for the samples reported here was from 1.33 to 1.82. The composition of chromatin from purified nuclei of P 815 cells had protein/DNA ratio of 1.50 (Prescott, 1977). The chromatin isolated from rat liver, which has been used as a model for chromatins in general, possessed a histone/DNA ratio of 1.0 and non-histone chromosomal protein to DNA in ratio of 0.6, or a combined ratio of total chomosomal protein to DNA of 1.6 (Bonner, 1979). A ratio substantially greater than 1.6 suggests contamination by nonchromosomal proteins adherent to the chromatin (Bonner, 1979).

(2) Time profile of specific retention in chromatin

The specific retention of TA in chromatin was investigated by isolating chromatin from intact nuclei, and the radioactivity was measured after the solubilization of chromatin with 0.01 SSC-glycerol. The TA

specifically retained in chromatin was normalized for equal amounts of DNA or chromosomal protein (Table XXI). The maximum retention of TA in chromatin, 1.1 fmole/μg DNA was seen to occur between 4 and 6 hours after incubation, while a decrease in the retention was observed at 24 hours, after which time the level was maintained up to 96 hours (Figure 43). Normalization of the radioactive data by equal amounts of chromosomal protein gave a similar profile of the specific retention of TA in chromatin (Figure 44) with the maximum retention of 0.6 fmole/μg chromosomal protein occuring between 4 and 6 hours after incubation.

The specific retention of ^{3}H-TA in chromatin over extended periods of incubation time as reported in this thesis has not been reported before. Middlebrook et al. (1975) and Aronow (1979) did find a tightly bound fraction of ^{3}H-TA in the nuclei of L-929 cells which could not be dissociated from the nuclei with 0.3 M KCl. They however worked with crude nuclear pellets rather than chromatin and thus only had a tentative basis for their suggestion that TA is retained within the chromatin. The time profile of the specific retention of the TA here described confirms that the uptake of ^{3}H-TA into the nuclei indeed results in a tight association with the chromatin, in the vicinity of DNA molecules, over the 96 hours examined.

(3) Intranuclear distribution of ^{3}H-TA

The distribution of nuclear TA between nucleoplasm and chromatin was also investigated. The TA retained in nucleoplasm was defined operationally as the fractions of radioactivity which could be removed by lysis of nuclear membrane with NaCl-EDTA (80 mM NaCl, 20 mM EDTA, pH 6.3) and subsequent wash with 0.01 SSC (1.5 mM NaCl, 0.15 mM Na_2-citrate pH 7.0). The radioactivity remaining in the gel-like chromatin pellet represented the TA retained in chromatin. The percent of nuclear TA in

Table XXI Specific retention of TA in chromatin

Incubation time, hr.	No. of samples[a]	Specific retention[b]	
		fmole/µg DNA	fmole/µg chromosomal proteins
0.5	9	0.052 ± 0.019	0.032 ± 0.007
2	6	0.436 ± 0.046	0.339 ± 0.025
4	6	1.065 ± 0.182	0.530 ± 0.026
6	9	1.025 ± 0.297	0.565 ± 0.026
24	6	0.747 ± 0.038	0.297 ± 0.083
48	3	0.712 ± 0.040	0.423 ± 0.033
72	3	0.728 ± 0.031	0.315 ± 0.021
96	6	0.726 ± 0.053	0.266 ± 0.012

(a) Number of samples of 1 - 3 independent experiments.

(b) Mean ± S.E.

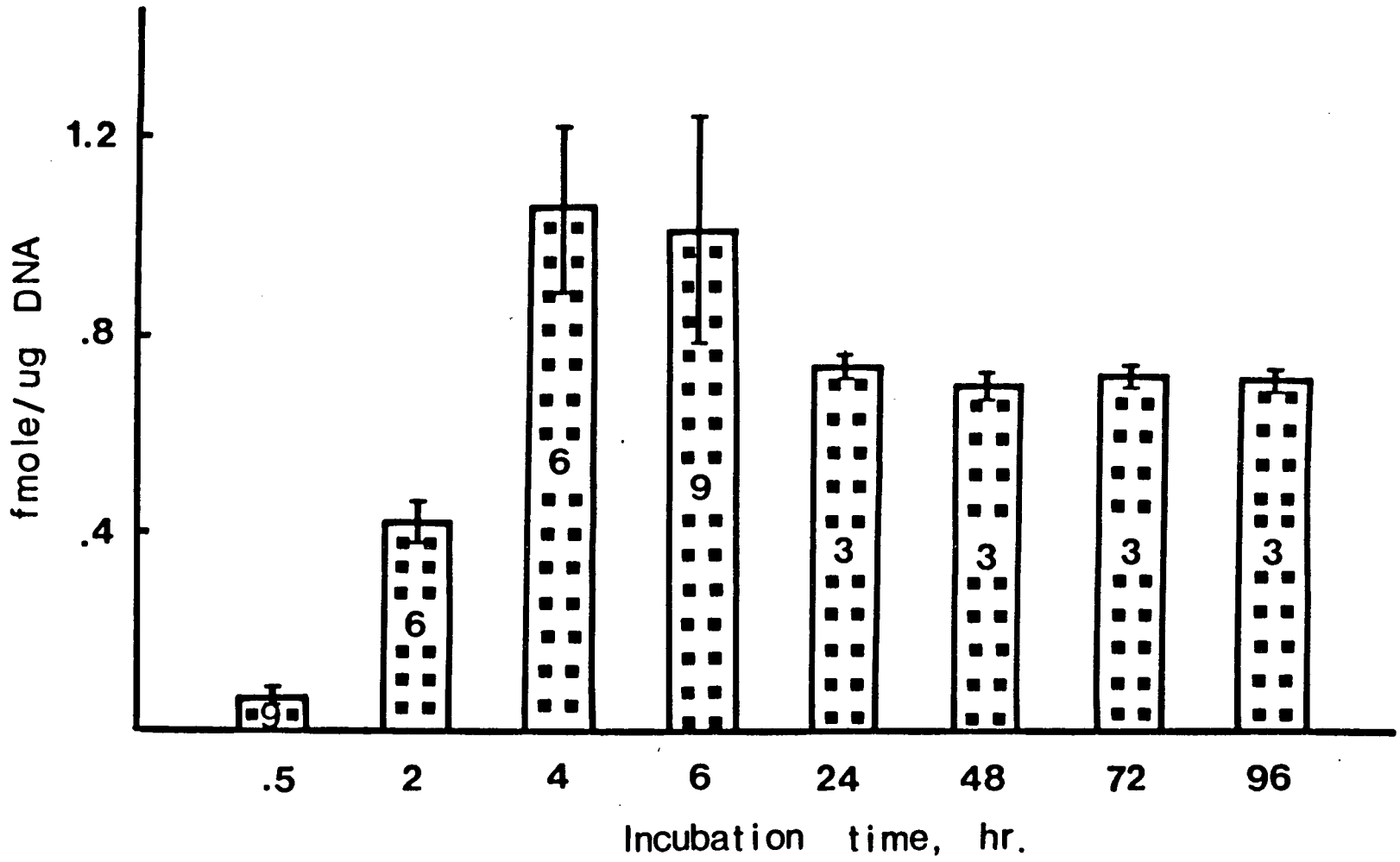


Figure 43 : Specific retention of TA in chromatin, normalized on DNA basis

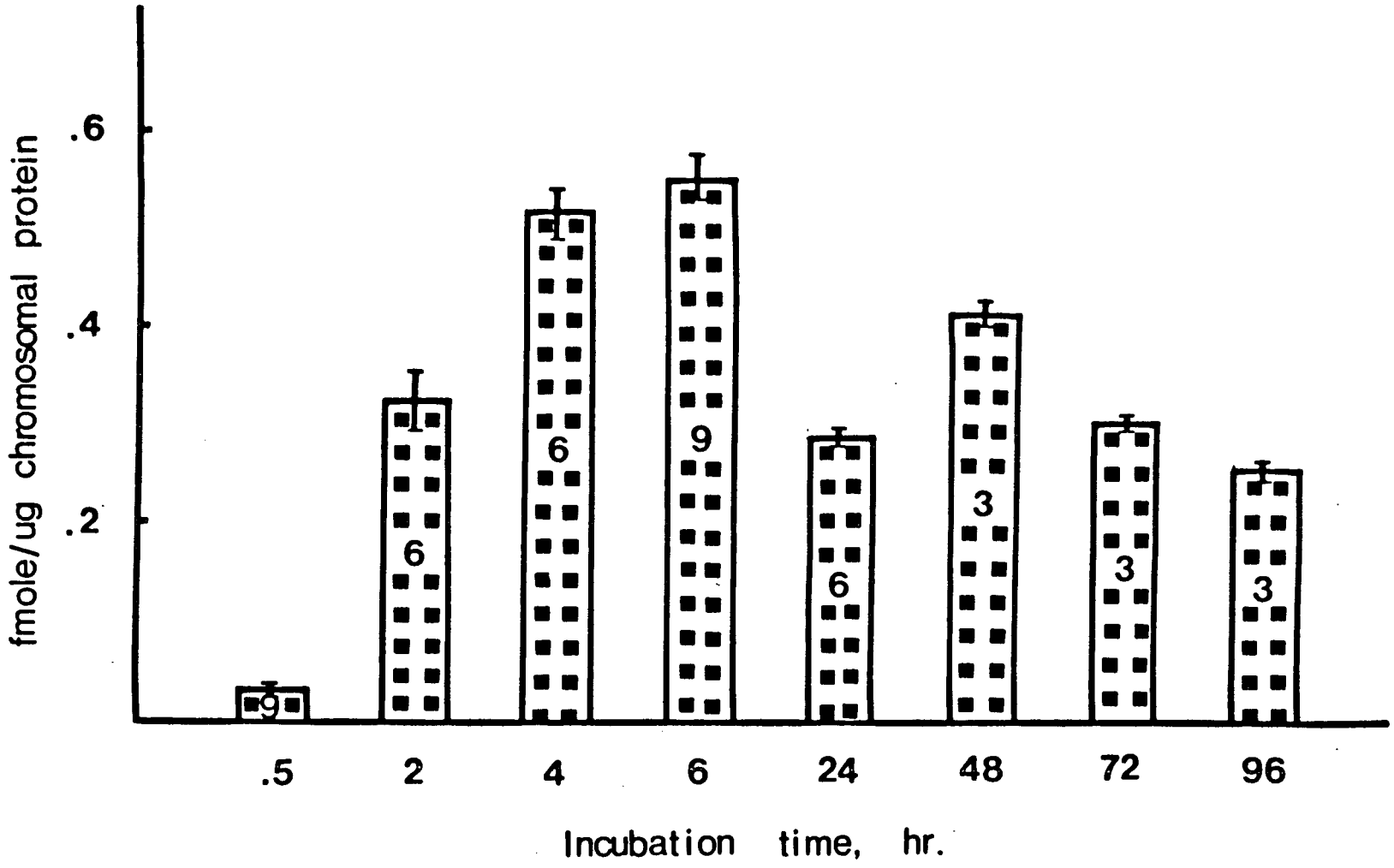


Figure 44 : Specific retention of TA in chromatin, normalized on chromosomal protein basis

chromatin was always more than 50%, ranging from 56.5 ± 6.4% to 73.9 ± 5.6% of total TA retained in nuclei, varying with the incubation times. The remaining TA was in nucleoplasm, ranging from 26.1 ± 5.6% to 43.5 ± 6.4% (Table XXII; Figures 45 and 46).

The incubation with unlabeled competitor caused the ^{3}H-TA uptake into nuclei to become negligible. Under these conditions, most nuclear TA was located in the nucleoplasm (71.2 ± 7.0%) for first 2 hours of incubation, and remained between 43.1 ± 6.4% and 47.9 ± 7.4% for the rest of incubation intervals.

Higgins et al. reported that when intact cells were exposed to steroids and cell nuclei were then fractionated, glucocorticoid-receptor complexes were found only in chromatin but not in the nucleolus, nucleoplasm and nuclear membrane, quoting their unpublished observations (Higgins et al., 1979). However, in our studies, chromatin only retained the majority of nuclear TA, 57-74%. With the remaining TA located in extrachomosomal compartments of the nucleus. Higgins et al. used different cell lines from ours for the study, which might account for this discrepancy.

(F) Subchromatin localization of ^{3}H-TA

A preliminary attempt was made using the technique of Bloom and Anderson (1978) to fractionate the ^{3}H-TA containing chromatin into various classes of histone and non-histone proteins by hydroxyapatite chromatography. The fractions of HAP eluates were monitored by both A_{230} measurement and liquid scintillation counting.

Specific dissociation patterns of total chromosome proteins are illustrated in Figure 47. It was obtained from chromatin immobilized on HAP as eluted with NaCl and NaCl plus urea solutions.

To examine the dissociation pattern of TA retained in chromatin,

Table XXII Intranuclear distribution of ^{3}H-TA between nucleoplasm and chromatin in the absence or the presence of 500-fold unlabeled TA

Incubation time, cpm	No. of samples	Without unlabeled TA			With 500-fold unlabeled TA		
		Total intranuclear activity, cpm	% distribution		Total intranuclear activity cpm	% distribution	
			Nucleoplasm	chromatin		Nucleoplasm	chromatin
0.5	9	704 ± 177	43.5 ± 6.4	56.5 ± 6.4	191 ± 66	71.2 ± 11.1	28.8 ± 11.1
2	6	7097 ± 1114	31.0 ± 0.8	69.1 ± 0.8	365 ± 185	71.2 ± 7.0	28.8 ± 7.0
4	6	9980 ± 470	26.1 ± 5.6	73.9 ± 5.6	280 ± 74	45.5 ± 3.0	54.5 ± 3.0
6	12	7944 ± 1268	27.0 ± 4.5	73.0 ± 4.5	397 ± 146	43.1 ± 6.4	56.9 ± 6.4
24	6	6525 ± 1846	33.2 ± 3.0	66.8 ± 3.0	291 ± 115	36.6 ± 3.3	63.5 ± 3.3
48	3	16564 ± 833	44.5 ± 2.3	55.5 ± 2.3	165 ± 43	46.1 ± 5.2	53.9 ± 5.2
72	3	14515 ± 680	24.7 ± 1.6	75.3 ± 1.6	406 ± 22	53.0 ± 2.9	47.0 ± 2.9
96	6	9315 ± 3569	32.6 ± 12.0	67.4 ± 12.0	593 ± 13	47.9 ± 7.4	52.1 ± 7.4

* data were presented as mean ± S.E.

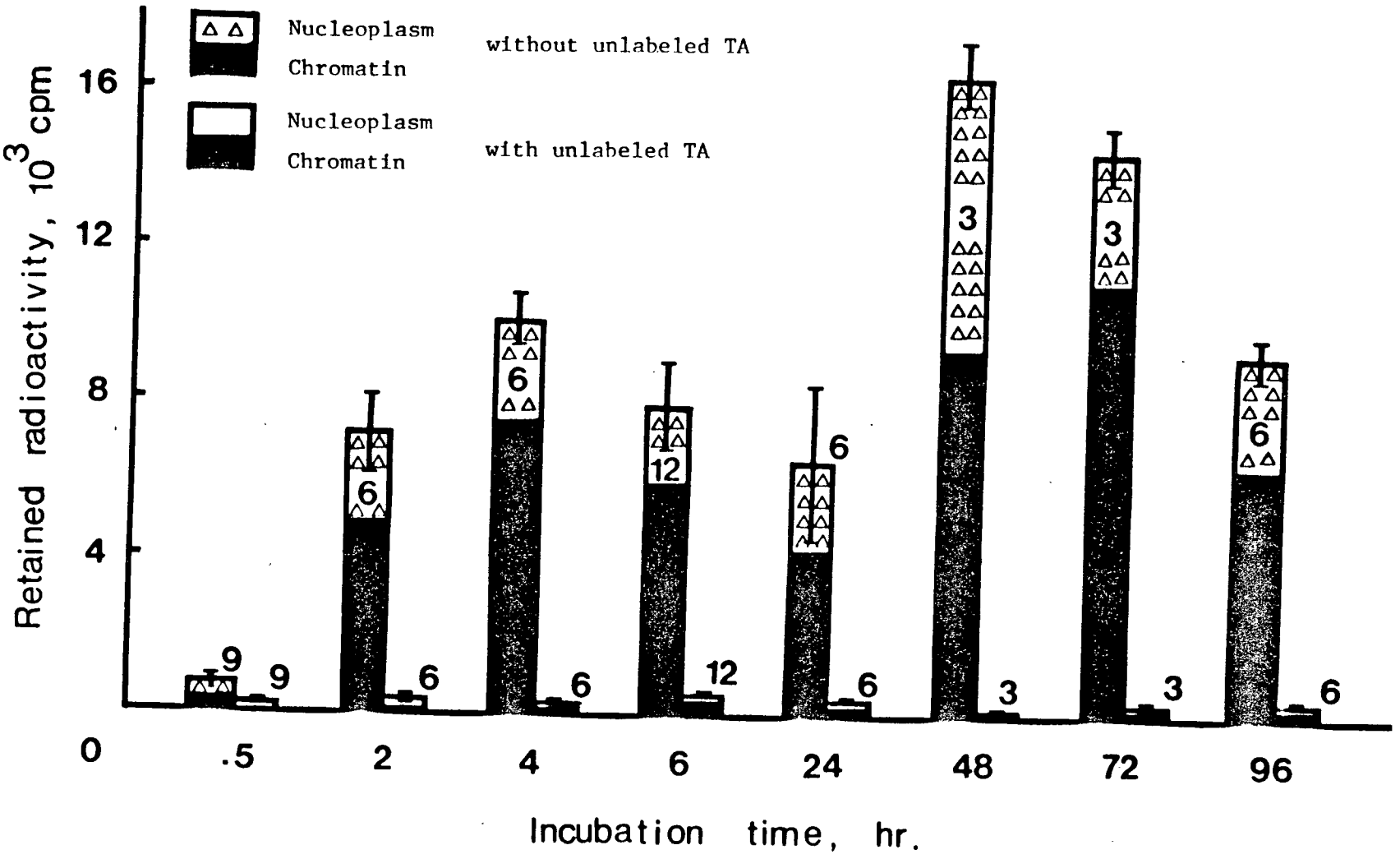


Figure 45 : Intranuclear distribution of ^{3}H-TA between nucleoplasm and chromatin in the absence or the presence of 500-fold unlabeled TA

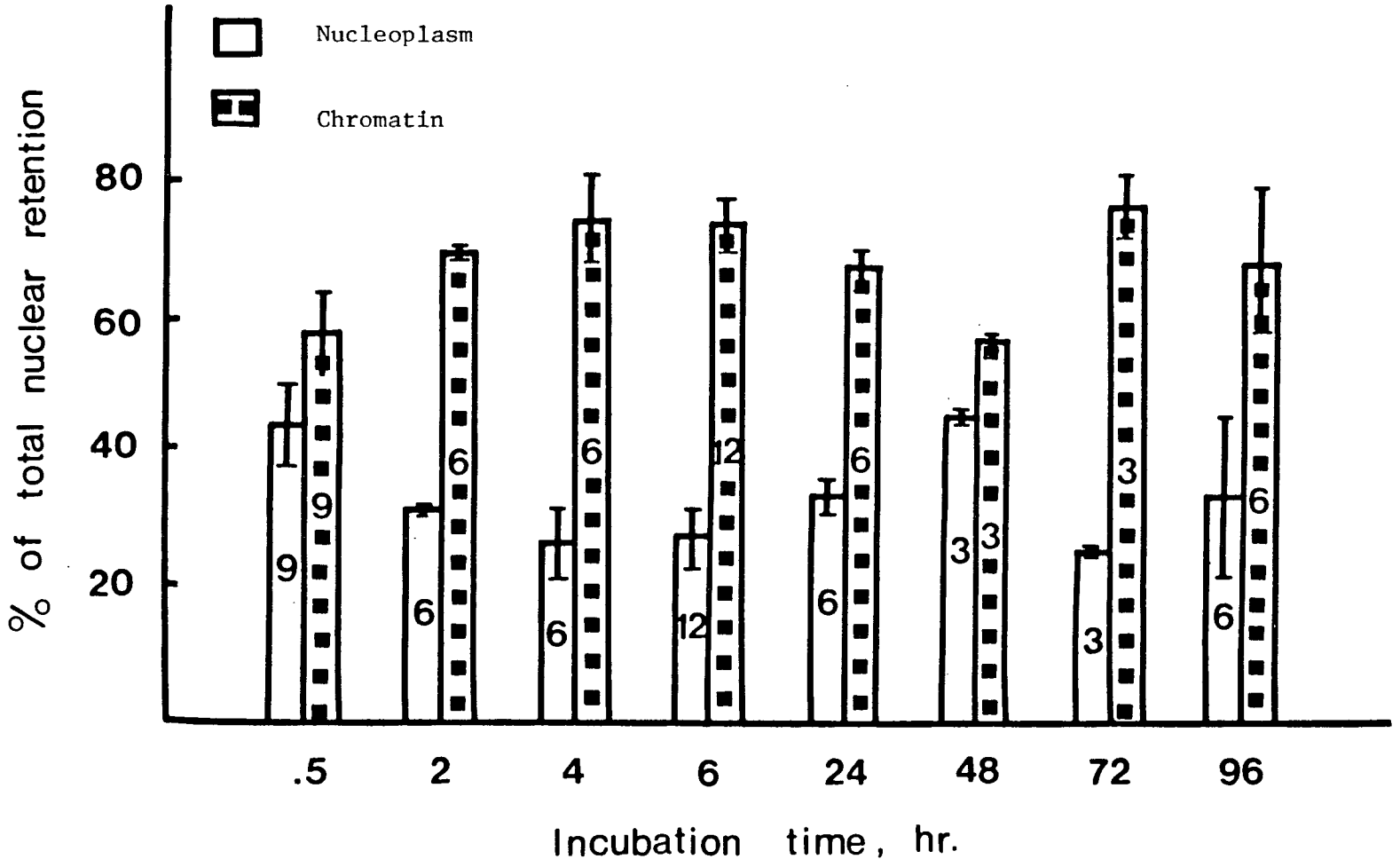


Figure 46 : Percent distribution of total nuclear ^{3}H-TA between nucleoplasm and chromatin

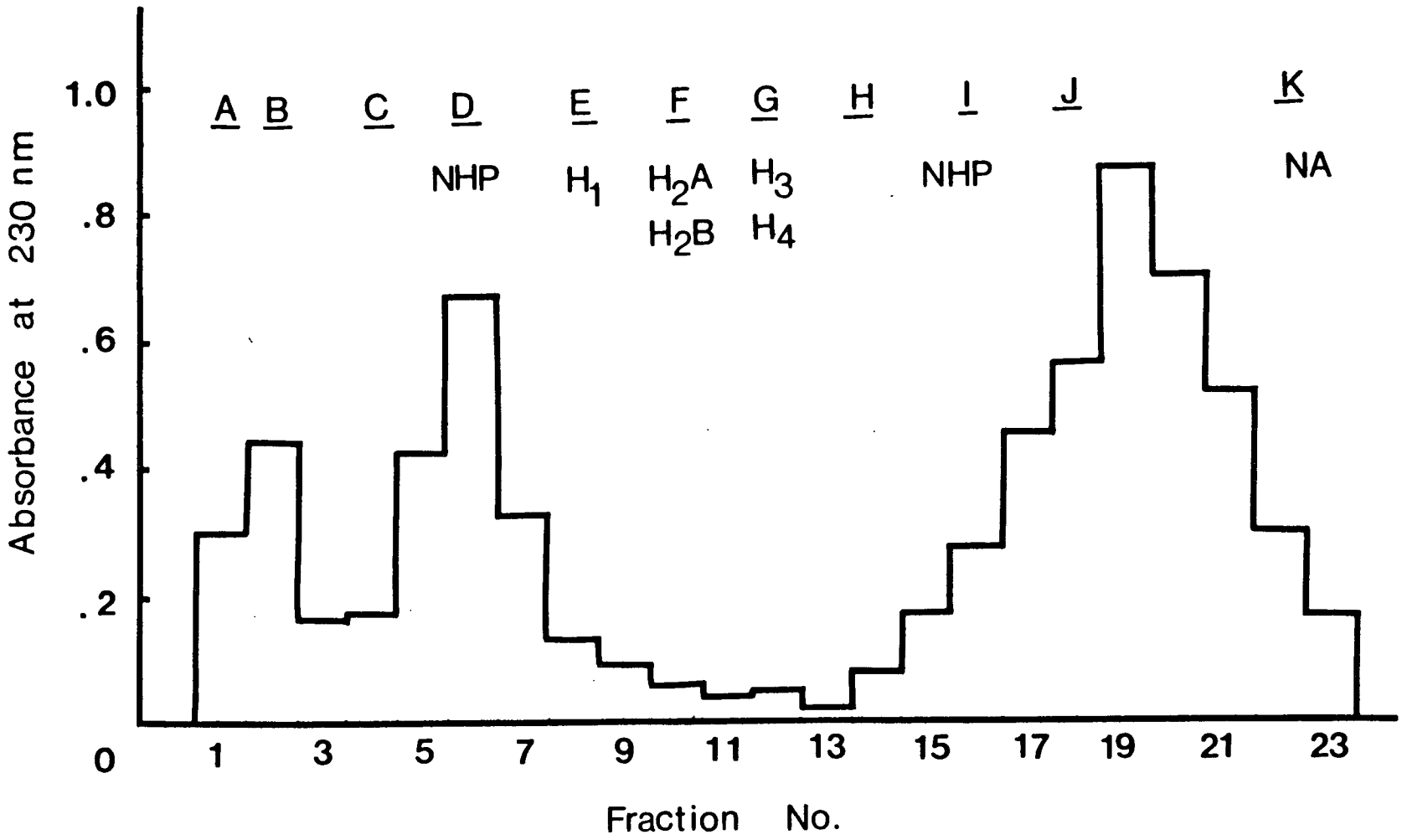


Figure 47 : Dissociation pattern of total chromosomal proteins from immobilized chromatin on HAP column as eluted with NaCl followed by NaCl and urea

Letters, A-K, NHP, NA, H_1, H_2A, H_2B, H_3 and H_4, are referred to Table V.

^{3}H-TA-containing and TA-free chromatin were chromatographed in a parallel fashion. Approximately 75% of the specifically bound TA was dissociated from the HAP-immobilized chromatin along with proteins designated by Bloom and Anderson (1978) as those of unbound chromosomal proteins (Fraction A, B and C, eluted with 0 to 0.25 M NaCl). Less than 13% of TA was dissociated along with histone proteins by increasing the NaCl concentration to 2 M (Fractions D, E, F and G). The remaining 12% was eluted along with the tightly bound non-histones by treatment with 2 M NaCl in the presence of urea (Fractions H, I and J) (Figure 48).

The tightly bound TA, reminiscent of the tightly bound estrogen receptor complexes reported by Bloom and Anderson were thought to be the nuclear residual forms of glucocorticoid. Bloom and Anderson (1978) proposed the interaction of the tightly bound estrogen as a direct association with DNA or possibly the association with acceptor proteins which in turn were bound tightly to the DNA (Spelsberg, 1974; Puca et al., 1975; Mainwaring et al., 1976).

Glucocorticoids are believed to share these possibilities with estrogens except that the direct association of cross-linking in case of glucocorticoid has been found unlikely to be present. Bugany and Beato (1977) proposed the possibility that the TA-receptor complexes recognize other components of the chromatin, such as the chromosomal proteins. Reports from other steroid target tissues have shown an interaction of the corresponding receptors with basic and acidic proteins of the chromatin (O'Malley et al., 1972; Puca et al., 1975). The data of this study supported the view of Bugany and Beato that the ^{3}H-TA which was specifically retained in chromatin, was largely associated with chromosomal proteins rather than with DNA.

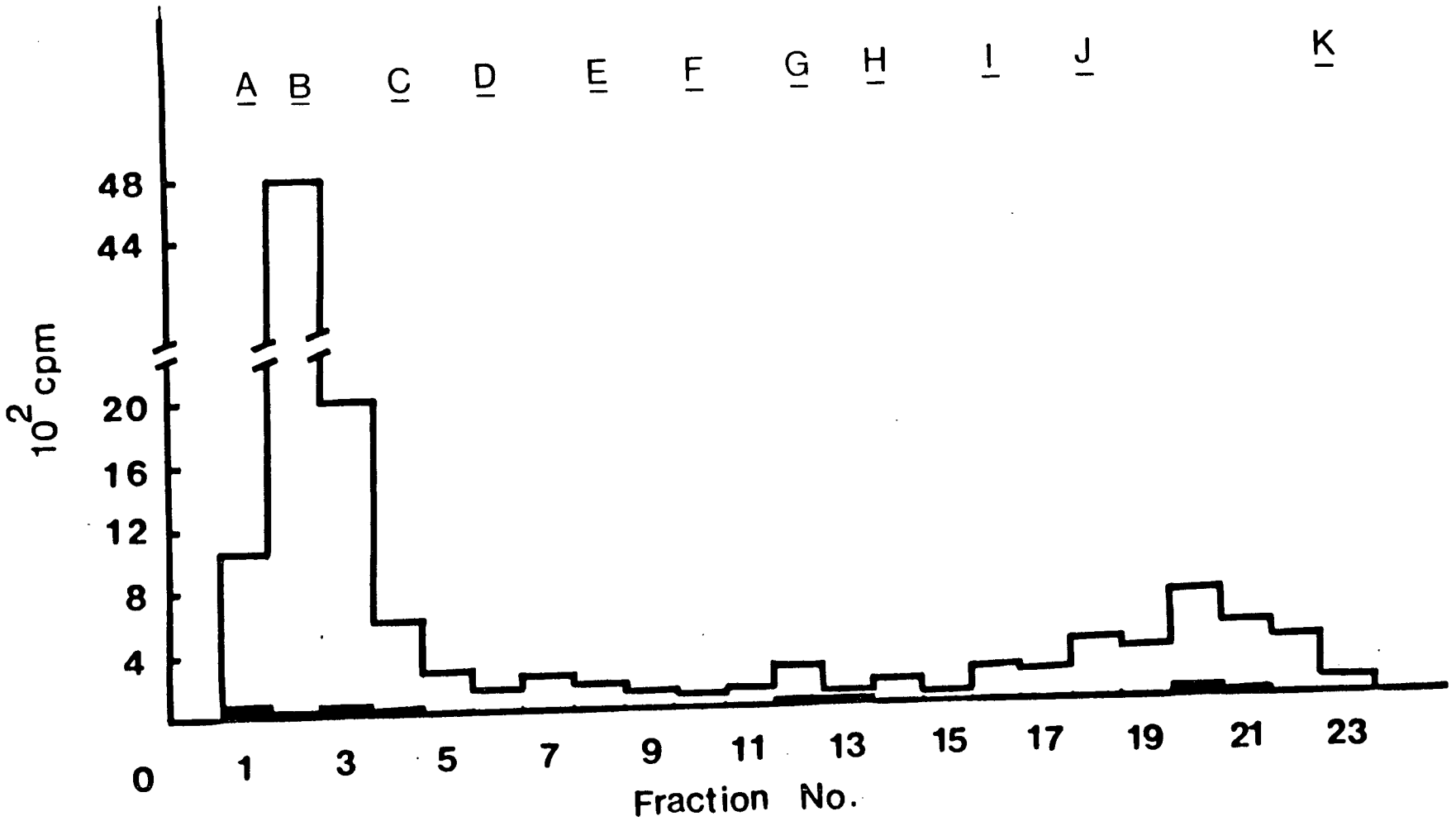


Figure 48 : Dissociation of ^{3}H-TA from chromatin as eluted with NaCl followed by NaCl and urea

Open histograms: without unlabeled TA

Shaded histograms: with 500-fold unlabeled TA

Letters of A-K are referred to Table V.

SUMMARY

(1) A gas-liquid chromatographic analytical technique was developed to quantitate hydrocortisone, triamcinolone acetonide and desonide levels in tissue culture media. The assay detects as little as 20 ng of the glucocorticoids.

The analysis of the three glucocorticoids in propylene glycol stock solutions has demonstrated that the glucocorticoids are stable upon storage.

The studies of the glucocorticoids in culture media in the presence of serum and fibroblasts have shown that the glucocorticoids are chemically stable with no detectable formation of metabolites under the conditions of incubation.

The concentrations of triamcinolone acetonide and desonide in medium of cell cultures fell 20% within 2 hr. Thereafter, the concentrations remained constant up to 84 hr. Therefore, for any biological effect of synthetic glucocorticoids observed at the dose of 1 ug/mL medium, the glucocorticoid responsible for the observed effect was not more than 20% of the dose.

The loss of HC from the media due to cells was more than 40%, larger than that of synthetic glucocorticoids. The HC also had relatively constant levels during the prolonged periods of incubation.

(2) The cross-linking of DNA with glucocorticoids was investigated by hydroxyapatite chromatography and thermal scanning analysis using (a) two cell lines, mouse L-929 and human dermal fibroblasts, (b) two glucocorticoids, HC and TA, (c) two glucocorticoid concentrations, 1 ug/mL medium and 10 ug/mL medium, (d) various times of incubation ranging up to 96 hours, (e) with or without UV-A irradiation.

No cross-linking was detectable under these experimental conditions.

(3) The uptake and retention of ^{3}H-TA into the nucleus and the effect of TA on the amount of nuclear proteins and DNA were determined. ^{3}H-TA was present in nuclei of cultured fibroblasts and suppressed the amount of nuclear proteins and DNA. Thus, the absence of cross-linking of DNA with glucocorticoids was not due to the failure of the translocation of TA into the nuclei, and was not due to the unresponsiveness of the cells to the glucocorticoids.

(4) The specific retention of triamcinolone acetonide, at a concentration of 10^{-8} M in culture media, reached the highest level, 0.4 fmole/ug nuclear protein or 2.8 fmole/ug DNA, in intact nuclei of mouse L-929 fibroblasts after 6 hours of incubation. Relatively constant,lower levels, 0.2 -0.3 fmole/ug nuclear protein or 1.0-1.1 fmole/ug DNA, were observed between 24 and 96 hours.

(5) Approximately 10% of cytosol-retained TA was translocated into the nucleus. The fraction translocated in the nucleus remained relatively unchanged up to 96 hours.

(6) The specific retention of triamcinolone acetonide, at a concentration of 10^{-8} M in culture media, reached the highest level, 0.6 fmole/ug chromosomal protein or 1.1 fmole/ug DNA, in chromatin of mouse L-929 fibroblasts after 6 hours of incubation. Relatively constant,lower levels, 0.3-0.4 fmole/ug chromosomal protein or 0.7 fmole/ug DNA, were observed between 24 and 96 hours.

(7) The intranuclear distribution of ^{3}H-TA, at the level of 10^{-8} M in culture medium, between nucleoplasm and chromatin was examined. The majority of TA in the nucleus, 56-75%, was associated with chromatin, and the remaining nuclear TA was located in nucleoplasm.

(8) A preliminary investigation of subchromatin localization of ^{3}H-TA was performed. Approximately 75% of the specifically retained TA was dissociated from the HAP-immobilized chromatin along with proteins designated by Bloom and Anderson as those of unbound chromosomal proteins. Less than 13% of TA was dissociated along with histone proteins. The remaining 12% was eluted along with the tightly bound nonhistone proteins. Virtually, no ^{3}H-TA was eluted with nucleic acid fractions; therefore, this study strengthens the view that glucocorticoids do not reach DNA in intact cells but, in the form of a glucocorticoid-receptor complex, exert their effects on DNA indirectly through some as yet to be characterized perturbation of the structure of nuclear proteins.

APPENDIX I

MATERIALS

1. ACETALDEHYDE, Cat. No. 468, Eastman Kodak Co., Rochester, New York.
2. ANTIBIOTIC-ANTIMYCOTIC MIXTURE (100 X), Penicillin 10,000 units/mL, Streptomycin 10,000 µg/mL, Fungizone 25 µg/mL, Cat. No. 600-5240, Gibco, Grand Island Biological Company, Grand Island, New York.
3. AQUASOL-2, Universal L.S.C. Cocktail, Lot. No. 229 AT9, New England Nuclear, Boston, Massachusetts.
4. BIO-RAD Protein ASSAY KIT, consisting of dye reagent (containing phosphoric acid and methanol) concentrate (control no. 18291) and protein standard (control no. 18329), Bio-Rad Laboratories, 32nd & Griffin, Richmond, California.
5. CALF THYMUS DNA, Type I, highly polymerized, Lot 48C-9580, Sigma Chemical Co., St. Louis, Missouri.
6. Ca^{++}, Mg^{++}-containing PBS (for photoreaction)

   0.05 M sodium phosphate buffer (pH 6.9)

   0.10 M NaCl

   0.001 M $MgCl_2$

   0.002 M $CaCl_2$
7. Ca^{++} and Mg^{++}-free PBS, (phosphate buffered saline), pH = 7.2.

KCl	0.2 gm/L
KH_2PO_4	0.2 gm/L
Na_2HPO_4	1.15 gm/L
NaCl	8.0 gm/L

Preparation: Soln A

KCl	4 gm/100 mL	(Lot BBR, Mallinckrodt)
KH_2PO_4	4 gm/100 mL	(Fisher, Lot 742804)

Soln B

Na_2HPO_4 23 gm/500 mL (Lot BNP, Mallinckrodt)

For making 1 L: 8 gm of NaCl + 5 ml of soln A + 25 ml of soln B, adjust pH, H_2O q.s.

8. DESONIDE, Lot No. CS-1-27, Dome laboratories, Division Miles Lab. Inc., West Haven, Connecticut.
9. DIALYSIS TUBING, Spectrapor® tubing, M.W. cutoff 12,000-14,000, cylind. dia. 6.4 mm., Spectrum Medical Industries Inc., Terminal Annex, Los Angeles, California.
10. DILUTE SALINE-CITRATE, DSC, 0.015 M NaCl and 0.0015 M sodium citrate pH 6.85.
11. DIPHENYLAMINE, Reagent ACS, Lost C8A, Eastman Kodak Co., Rochester, New York.
12. DISODIUM ETHYLENEDIAMINETETRAACETATE (Na_2-EDTA), Lot 770619, Certified A.C.S., Fisher Scientific Co., Fair Lawn, New Jersey.
13. DULBECCO'S MODIFIED EAGLE MEDIUM (DMEM), Powder, Cat. No. H-16, Gibco, Grand Island Biological Company, Grand Island, New York.
14. ETHYL ACETATE, distilled in glass, Caledon, Georgetown, Ontario.
15. FETAL CALF SERUM, mycoplasm tested and virus screened, Cat. No. 200-6140, Gibco, Grand Island Biological Company, Grand Island, New York.
16. GLACIAL ACETIC ACID, Reagent A.C.S., Code 001019-005-59-0, Allied Chemical Canada Ltd., Pointe Claire, Quebec.
17. HYDROCORTISONE, Lot No. 402-9511, Pfizer Co. Ltd., Montreal.
18. HYDROXYAPATITE, DNA grade, Bio-Gel® HTP, Control No. 19350, BioRad Laboratories, Richmond, California.
19. HYPOTONIC SOLUBILIZING BUFFER, pH 7.4
consisting of 20 mM Tris-HCl (Tris (hydroxymethyl) aminomethane,

J.T. Baker Chem. Co., N.J.)

2 mM $CaCl_2$ (Fisher)

2 mM $MgCl_2$ (J.T. Baker Chem. Co. N.J., Lot 601-6809)

3 mM dithiothreitol (Lot 117C-0287, Sigma, stored at 0-5°C in dessicator)

5% glycerol

20. METHANOL, distilled in glass, Caledon, Georgetown, Ontario.

21. METHOXYAMINE HYDROCHLORIDE, Lot # 04239.19, Pierce Chemical Co., Rockford, Illinois.

22. 8-METHOXYPSORALEN, 8-MOP, Lot 34C-1660, solution in 50% EtOH, 100 µg/mL, Sigma, St. Louis, Missouri.

23. MOUSE L-929 DERMAL FIBROBLASTS, Passage No. of 578, 584 and 564, Flow Laboratories, McLean, Virginia.

24. N-TRIMETHYLSILYL IMIDAZOLE, Lot # 0211774, 10 x 1 gm ampoules, Pierce Chemical Co., Rockford, Illinois.

25. PERCHLORIC ACID, $HClO_4$, 60%, analytical reagent, Lot ENV, Mallinckrodt Inc., St. Louis, Missouri.

26. PROGESTERONE, Lot No. 113C-0190, Sigma Chemical Co., St. Louis, Missouri.

27. PROPYLENE GLYCOL, Laboratory grade, Fisher Scientific Co., Fair Lawn, New Jersey.

28. PYRIDINE, Silylating grade, Pierce Chemical Co., Rockford, Illinois.

29. REACTI-VIAL, Pierce Chemical Co., Rockford, Illinois.

30. SALINE/EDTA, pH = 7.4

consisting of 100 mM NaCl (American Scientific & Chemical, Lot 63719)

10 mM Na_2-EDTA (Certified ACS. Fisher Scientific Co., Lot 770619)

31. SODIUM BICARBONATE, analytical reagent grade, Mallinckrodt Inc., St. Louis, Missouri.

32. SODIUM CHLORIDE, certified A.C.S., Lot 766294, Fisher Scientific Co., Fair Lawn, New Jersey.

33. SODIUM CITRATE, tribasic, Lot 80029, analytical reagent, BDH Chemicals, Toronto.

34. SODIUM DODECYL SULFATE (S.D.S.), Lot 772002, Laboratory grade, Fisher Scientific Co., Fair Lawn, New Jersey.

35. SODIUM HYDROXIDE, Lot 764282, Certified A.C.S., Fisher Scientific Co., Fair Lawn, New Jersey.

36. SODIUM PHOSPHATE BUFFER, pH 6.8,

    0.5 M (0.293 M Na_2HPO_4 and 0.207 M $NaH_2PO_4 \cdot H_2O$)

    0.24 M (dilute 480 mL of 0.5 M buffer to 1 L)

    0.01 M (dilute 20 mL of 0.5 M buffer to 1 L)

    SODIUM PHOSPHATE, MONOBASIC, monohydrate, granular, Code 2312, SODIUM PHOSPHATE, DIBASIC, anhydrous, Lot 5074, B & A, Allied Chemical, General Chemical Div., Pointe Claire, Quebec.

37. STANDARD SALINE-CITRATE, SSC, 0.15 M NaCl and 0.015 M sodium citrate pH 6.85.

38. SULFURIC ACID, CONCENTRATED 93-98%, Fisher Scientific Co., Fair Lawn, New Jersey.

39. t-BUTYLDIMETHYLSILYL CHLORIDE MIXTURE, contining 1.0 mmole t-butyldimethylsilyl chloride, 2.5 mmole imidazole per mL of anhydrous N,N-dimethylformamide, Lot # 18028, Applied Sciences Laboratories Inc., Pennsylvania.

40. TISSUE CULTURE DISH, 3003 Optilux®, 100 x 20 mm Style Dish, Falcon, Div. Becton, Dickinson an Co., Cockeysville, Maryland.

41. TRIAMCINOLONE, USP, Code #2014, Batch # Z5040, Cyanamid of Canada Ltd., Montreal.

42. TRIAMCINOLONE ACETONIDE, USP, Code #45645, Batch #431, Cyanamid of Canada Ltd., Montreal.

43. TRITIATED-HYDROCORTISONE ^{3}H-HC, Batch 38, specific activity 42 Ci/mmole in benzene/EtOH (9:1), Amersham/Searle, Arlington Heights, Illinois.

44. TRITIATED-TOLUENE STANDARD, Code No. 188280, Lot H177, activity 1.297 x 10^{6} dpm/g ± 0.2%, Date October 1/77, Amersham, Arlington Heights, Illinois.

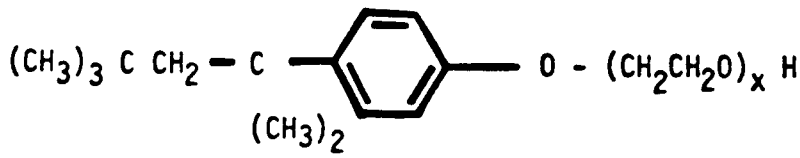
45. TRITIATED-TRIAMCINOLONE ACETONIDE ^{3}H-TA,

(a) NET-470, Lot 853-017, specific activity 2.16 Ci/mmol in EtOH,

(b) NET-470, Lot 998-277, specific activity 31.3 Ci/mmol in benzene/EtOH (9:1),

New England Nuclear Co., Boston, Massachusetts.

46. TRITON X-100, Lot 87C-0075, polyethylene glycol p-isooctylphenyl-ether (alkyl polyether alcohol) d_4^{25} = 1.0595, non-ionic detergent, Sigma, St. Louis, Missouri.



47. TRYPSIN, sterile, 10X, 2.5% in modified Hanks' balanced saline solution, without Mg & Ca, Lot 16893026, Flow Laboratories, McLean, Virginia.

48. UREA, Lot 12G08, prilled, reagent A.C.S., Matheson Coleman & Bell Manufacturing Chemists, Norwood, Ohio.

APPENDIX II

APPARATUS

1. AMICON FILTRATION UNIT, Stirring filtration unit, Model 12, and Diaflo XM 100 A membrane, Amicon Co., Lexington, Massachusetts.
2. BECKMAN DB-GT SPECTROPHOTOMETER and 10" linear recorder equipped with 1P28A photomultiplier, deuterium and tungsten sources, Beckman Instruments, Inc., Fullerton, California.
3. BENCH-TOP CLINICALCENTRIFUGE, Cat. No. 809, AC or DC, with build-in fixed angle rotor, International Equipment Co., Boston, Massachusetts.
4. BLACK LIGHT BLUE FLUORESCENT LAMP, UV-A 330 (310-360 nm), 0.6 mW/cm^2 in intensity, Sylvania F20T12-BLB, Toronto, Ontario.
5. CARBON DIOXIDE-CELL INCUBATOR, Model 3221-14, National Appliance Co., (a Heinicke Co.), Portland, Oregon.
6. CALCULATOR, Model HP-97, Program SD-03A, Hewlett-Packard, Avondale, Pennsylvania.
7. CONSTANT TEMPERATURE BATH, HAAKE, Model E-51, Gehruder Haake K.G., Berlin, Germany.
8. DIFFERENTIAL SCANNING CALORIMETER, Model 1B, Perkin-Elmer, Norwalk, Connecticut.
9. GAS-CHROMATOGRAPH-ELECTRON-IMPACT MASS SPECTROMETER, Varian MAT-111, Varian, Pala Alto, California.
10. GAS-LIQUID CHROMATOGRAPH, Model 5830A, equipped with a terminal, Model 18850A, Hewlett-Packard, Avondale, Pennsylvania.
11. GILSON MICRO FRATIONATOR, equipped with sensors for time or drops, Terochem. Laboratories Ltd., Edmonton, Alberta.

12. GRADIENT MIXER, Model GM-1, consisting of two reservoir cylinders of 300 mL capacity and low speed, 250 rpm synchronous motor, Pharmacia Fine Chemicals, Sweden.
13. HEMACYTOMETER, Neubauer improved, Albert Sass.
14. INVERTED MICROSCOPE, with attached camera (100 X, 200 X, 400X), Nikon 44012, Japan, Nikon M-35S camera, Nikon 38527 light source, Nikon 4FM camera adapter.
15. ISOCAP/300 PROGRAMMABLE LIQUID SCINTILLATION SYSTEM, Model 6868, ambient temperature, Nuclear Chicago, Des Plaines, Illinois.
16. MELTING POINT DETERMINATION APPARATUS, Thomas-Hoover, Arthur H. Thomas Co., Philadelphia, Pennsylvania.
17. PERISTALTIC PUMP, Desaga Model 131900, STA multipurpose, Brinkman, Heidelberg.
18. PHASE-CONTRAST MICROSCOPE, 150X, M40-58916, Wild Heerbrugg, Switzerland.
19. RADIOMETER pH METER, Type PHM 26 equipped with standard glass and calomel electrodes and built-in precision scale expander, Radiometer A/S, Copenhagen, Denmark.
20. SORVALL SUPERSPEED AUTOMATIC REFRIGERATED CENTRIFUGE, RC2-B, equipped with fixed-angle rotor SM-24 of r(radius) = 4.34 inches, Sorvall, Norwalk, Connecticut.
21. TEFLON-FITTED GLASS HOMOGENIZER, A.H.T. Co., Philadelphia, Pennsylvania.
22. UV-VIS SPECTROPHOTOMETER, Gilford, Model 250, equipped with automatic cuvette thermoprogrammer, Model 2527, Automatic reference compensator and cuvette selector, Model 2535, Analog multiplexer, Model 6046, Recorder, Model 6050, Oberlin, Ohio.
23. VORTEX-GENIE, Model 3,061,280, 10 speed controls, Fisher Scientific Co., Fair Lawn, New Jersey,

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