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QUANTITATIVE CYTOCHEMICAL STUDIES ON THE RELATION OF DEOXYRIBONUCLEIC ACID OF CELLS TO VARIOUS PATHOLOGICAL CONDITIONS*

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Résumé—Nous avons porté notre attention sur l'étude quantitative des acides désoxyribonucléiques, par la microspectrophotométrie sur réaction de Feulgen, dans des cellules anormales et dans des cellules placées dans des conditions pathologiques. Pour certains cas, nous avons également envisagé d'autres constituants cellulaires, comme des protéines, en utilisant la même technique mais sur réaction de Millon, ainsi que la microscopie en interférence.

Le présent rapport concerne principalement le comportement quantitatif des ADN dans les quatre circonstances pathologiques suivantes: (1) le stress chirurgical, (2) les tumeurs, (3) l'infection à virus et (4) l'infertilité. Elles ont été retenues parce que chacune d'elles met en cause des fonctions et systèmes cellulaires différents; pour chacune d'elles aussi le comportement des ADN est différent.

(1) Des chiens ont été gastrectomisés et cholécystectomisés; la quantité d'ADN a été mesurée à l'échelle cellulaire dans le foie et la surrénale. Cette dernière est intéressante à considérer car on sait le rôle qu'elle joue dans la réponse du sujet au stress; en outre, l'utilisation de la microspectrophotométrie est spécialement favorable à l'étude de cette glande car ses différentes zones peuvent être envisagées individuellement d'une cellule à l'autre, non seulement du point de vue cytochimique mais aussi du point de vue morphologique. L'étude de plus de 7000 cellules hépatiques et surrénaliennes de dix-sept chiens a révélé que le contenu en ADN reste inchangé au cours du stress. Cette stabilité des ADN est d'autant plus remarquable que, dans ces mêmes cellules, la tyrosine augmente. Le fait que ADN et tyrosine se comportent différemment au cours du stress chirurgical chez le chien concorde avec les résultats obtenus chez d'autres espèces et dans d'autres tissus présentant des anomalies différentes.

(2) Une vaste étude a été entreprise, qui porte à la fois sur des tissus humains cancéreux et sur des tissus humains normaux; pour ces derniers, en effet, peu de choses encore sont connues en ce qui concerne leurs ADN. Des mesures microspectrophotométriques ont été faites sur près de 10 000 cellules considérées individuellement dans septante et un tissus normaux et dans cinquante-deux tissus cancéreux. Quelles que soient leur origine ou leur fonction métabolique, les cellules de tous les tissus normaux contiennent une quantité moyenne d'ADN semblable de l'une à l'autre, ce qui concorde avec d'autres observations, faites sur des tissus d'animaux. Dans les cellules de tumeurs ou de lésions précancéreuses, au contraire, la quantité d'ADN est augmentée et les valeurs mesurées sont nettement plus dispersées. Ce fait ne peut cependant être considéré comme un critère spécifique de la transformation cancéreuse des cellules; il est explicable surtout par la croissance et l'activité mitotique élevées des tumeurs. Au contraire, puisque la plupart des tissus humains normaux ne montrent pratiquement pas de mitoses et

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ont, de ce fait, une quantité constante d'ADN, une augmentation de ces derniers et une dispersion plus grande des valeurs mesurées permettent de soupçonner de la malignité sauf si, bien entendu, des phénomènes de régénération sont attendus. Dans certains cas, en outre, les mesures quantitatives d'ADN permettent de faire la distinction, pour un même organe, entre une tumeur maligne et une tumeur bénigne. Ainsi, dans le cas de la prostate, les teneurs en ADN sont élevées s'il y a malignité, tandis qu'elles restent normales et constantes s'il s'agit d'une tumeur bénigne. Cette différence est intéressante à considérer car, dans les deux types de lésion, la taille des noyaux est augmentée, ainsi que les protéines intracellulaires; en outre, dans les deux cas également, des figures mitotiques sont rarement observées.

Des tumeurs animales spontanées ou transplantées ont également été étudiées. D'une manière générale, il n'y a pas de différence essentielle dans le comportement des ADN entre les tumeurs humaines et les tumeurs animales. Cette similitude est intéressante car les tumeurs que nous avons étudiées chez l'animal sont des tumeurs à origine virale connue.

(3) Différents types de cellules infectées de virus contenant de l'ADN ont été étudiées *in situ* chez l'hôte ou cultivées *in vitro*. Les noyaux de pareilles cellules renferment des configurations bizarres de masses d'acide désoxyribonucléique; parallèlement, on observe une augmentation et une variabilité frappantes des teneurs en ADN. Cette réponse, presque spécifique des cellules infectées de virus à ADN, est retrouvée quel que soit le type de cellule étudié (cellules humaines, cellules d'insectes, cellules normales ou cancéreuses) et qu'il s'agisse de cellules cultivées *in vitro* ou *in situ* dans l'organisme. Comme les cellules infectées ne se divisent pas, les quantités élevées d'ADN qu'elles contiennent ne peuvent être expliquées par un rythme rapide de division, comme c'est le cas pour les tumeurs. Il n'est pas possible, à l'heure actuelle, de déterminer si les taux élevés d'ADN sont dus à la multiplication du virus lui-même, ou à une synthèse anormale d'ADN par la cellule hôte, ou encore à ces deux possibilités combinées; les faits suggèrent cependant qu'il s'agirait plutôt d'une propagation virale. Il est intéressant de constater qu'aucun des virus contenant de l'ARN que nous ayions étudié jusqu'ici n'a d'effet similaire.

(4) Plus de 32 000 cellules spermatiques de 200 hommes et de plus de 100 taureaux ont été étudiées. Alors que la valeur haploïde d'ADN dans les spermatozoïdes de mâles fertiles est remarquablement constante et uniforme, la quantité d'ADN dans les spermatozoïdes de mâles infertiles est, au contraire, fréquemment variable et significativement plus basse. Cependant, l'aspect cytologique et le poids sec de ces spermatozoïdes ne sont pas modifiés, que ces derniers contiennent ou non une quantité normale d'ADN. Les mêmes constatations restent valables pour les cellules des lignées spermatiques; ainsi, chez des hommes infertiles, les spermatocytes I et II, de même que les spermatides sont déficients en ADN; cependant, l'aspect morphologique de ces cellules peut être tout à fait normal. Dans l'ensemble donc, la déficience en ADN, telle que la met en évidence la microspectrophotométrie sur réaction de Feulgen dans des cellules spermatiques d'apparence normale, peut être considérée comme un test de diagnostic et le critère d'au moins un type d'infertilité masculine.

INTRODUCTION

THE investigation of this problem was a co-operative effort in which numerous investigators in our laboratory, representing various branches of biology, chemistry, and medicine, participated.

In recent years, microscopists have become increasingly aware of the necessity of extending their light-microscope studies of tissues and cells into the chemical sphere. Although structural studies have yielded a wealth of information on morphological alterations, microscopists have recognized the limitations imposed

by purely structural studies, even if performed at the level of the electron microscope.

Chemical studies of intracellular substances in single cells, in which the morphological architecture of the cells and their relationship within a tissue are still preserved, were carried out by CASPERSSON in 1936. By combining the microscope with a photometric device which permitted light-absorption measurements of intracellular substances, he developed the method of microspectrophotometry (CASPERSSON, 1936, 1950). The basic principle of microspectrophotometry is simple: As in the photometric chemical analysis of solutions, the amount of light absorbed at a specific wavelength by an unstained or stained cell structure is used as a basis for the qualitative and quantitative analysis of the intracellular substance. This approach opened new pathways for the study of intracellular substances and stimulated the development of other methods, such as Feulgen microspectrophotometry and interference microscopy, because the microscope could now be used not only as the conventional tool for the study of morphology but, simultaneously, as an instrument for the chemical analysis of cell structures (POLLISTER and ORNSTEIN, 1955; LEUCHTENBERGER, 1957a).

The unique feature of these methods lies not only in the possibility that, for instance, protein and deoxyribonucleic acid (DNA) quantity can be determined in a single nucleus, such as that of a sperm, but that this analysis can be done in microscopic sections *in situ*. In other words, since the architecture of the cells and their relationship within a tissue are preserved, a direct comparison between cell morphology, protein and DNA content from cell to cell can be made directly under the microscope. Thus the DNA and protein behaviour can be explored under normal and abnormal conditions during such dynamic processes as growth, mitosis and meiosis. Such problems as those concerning the alterations in the DNA content associated with the formation of germ cells or the sequential DNA changes taking place after infection with a virus, as well as many others that pose questions formerly unanswerable because of lack of method, can now be solved.

The conventional biochemical analysis is obviously not suitable for exploration of such problems, because the biochemical DNA determination can be carried out at present only on relatively large cell populations. Although such an average DNA value may be representative for the cell type if the cell suspension analysed is one in which each cell has the same uniform DNA content, it is actually an erroneous value if the analysis is done on cells with varying DNA content, which is the case in tissues undergoing mitosis or meiosis and during many abnormal processes.

The possibility of a direct correlation between the functional stage of a cell, be it normal or abnormal, its microscopical appearance and its chemical composition assumes particular significance when one takes into consideration the fact that, in the final analysis, a change in function and morphology of a cell is more or less the expression of underlying, and often preceding, chemical alteration. The detection of intracellular chemical changes before structural alterations manifest themselves is, indeed, one of the intriguing advantages of microspectrophotometry and should be of great help to anyone attempting to explore the role of DNA and its relation to the occurrence of abnormalities (LEUCHTENBERGER, 1957b; LEUCHTENBERGER and LEUCHTENBERGER, 1957).

The study of intracellular substances and their relation to structural changes of cells and tissues under abnormal conditions has been the centre of our interest for over 10 years. Since the cell can be accepted as occupying a fundamental position and since it probably is one of the primary targets hit by injurious agents in disease, correlated morphological and chemical studies, using microspectrophotometry and interference microscopy, seemed to us to be of special importance. We felt that such simultaneous morphological and chemical investigation at the single-cell level might not only throw light on aetiology and pathogenesis but might also help greatly in the detection and understanding of disease.

In view of the important role of DNA for cell life and cell continuity, its quantitative stability under normal conditions and its close relationship to the genes, we focused our special attention on the study of DNA in abnormal cells and in cells under pathological conditions.

RESULTS

During the past 10 years we have investigated extensively, by means of Feulgen microspectrophotometry, the quantitative behaviour of DNA in cells in a wide variety of pathological conditions. We have also attempted to study in some of these processes other intracellular constituents, such as proteins, using particularly

TABLE 1. QUANTITATIVE BEHAVIOUR OF DNA AND OF PROTEINS FREQUENTLY ENCOUNTERED IN CELLS OF PATHOLOGICAL PROCESSES

	Pathological process	DNA	Protein
(1)	Surgical stress	Unchanged	Increased
(2)	Prostatic hypertrophy and hyperplasia	Unchanged	Increased
(3)	Precancerous lesions and benign tumours	Increased due to mitosis, polyploidy or polyteny	Increased
(4)	Malignant tumours (including tumours of known viral aetiology, such as Polyoma and Rous tumours)	Increased due to mitosis, polyploidy or polyteny	Increased
(5)	Bronchitis after exposure to cigarette smoke		
	(a) Early bronchitis	Unchanged	Increased
	(b) Bronchitis associated with proliferation	Increased due to mitosis, polyploidy or polyteny	Increased
(6)	Viral infections due to DNA-containing viruses	Increased	Not studied
(7)	Focal hepatic changes (after injection with mouse tumour DNA)	Increased	Not studied
(8)	Male infertility	Decreased	Unchanged
(9)	Dwarfism (including hereditary pituitary dwarfs)	In some tissues: decreased In some tissues: unchanged	Not studied
(10)	Early radiation effects	Decreased	Not studied
(11)	Early pycnosis	Unchanged	Decreased
(12)	Late pycnosis	Decreased	Decreased

Millon microspectrophotometry and interference microscopy (POLLISTER and ORNSTEIN, 1955; LEUCHTENBERGER, 1957a). The quantitative behaviour of DNA and of proteins frequently observed in cells of various pathological processes is summarized in Table 1. Information concerning species, types of tissues and cells

TABLE 2. PATHOLOGICAL PROCESSES WITH DNA QUANTITIES NOT SIGNIFICANTLY DIFFERENT FROM THOSE OF NORMAL RESTING HOMOLOGOUS CELLS

Pathological process	Species	Type of tissue or cell	Number of cells analysed	Range of DNA values in cells in basic units		References
				Abnormal	Normal	
Surgical stress	Dog	Liver	Over 3000	2	2	COLE and LEUCHTENBERGER, 1956 a, b; COLE *et al.*, 1958
		Adrenal	Over 4000	2	2	
Hereditary pituitary dwarfism	Mouse	Kidney	Over 400	2	2	LEUCHTENBERGER *et al.*, 1954a
		Spermatid	Over 400	1	1	
Early bronchitis (after exposure to cigarette smoke)	Mouse	Bronchus	Over 2500	2	2	LEUCHTENBERGER *et al.*, 1958a
		Lung	Over 2500	2	2	
Early stage of pycnosis	Mouse	Liver	Over 100	2, 4	2, 4	LEUCHTENBERGER, 1950
		Tumour	Over 100	2–4	—	
Dwarfism	Cattle	Liver	Over 400	2, 4	2, 4	LEUCHTENBERGER *et al.*, 1956d
Prostatic hypertrophy and hyperplasia	Human	Prostatic gland	Over 800	2	2	PERSKY and LEUCHTENBERGER, 1957

TABLE 3. PATHOLOGICAL PROCESSES WITH DNA QUANTITIES FREQUENTLY IN EXCESS OF THOSE OF NORMAL CELLS, BUT WITH THE INCREASE EXPLAINABLE ON THE BASIS OF MITOSIS, POLYPLOIDY OR POLYTENY

Pathological process	Species	Type of tissue or cell	Number of cells analysed	Range of DNA values in cells in basic units		References
				Abnormal	Normal in mitosis	
Atypical proliferation	Mouse	Bronchus	Over 10,000	2–4	2–4	LEUCHTENBERGER *et al.*, 1958a
Precancerous lesions	Human	Skin	Over 500	2–8	2–4	LEUCHTENBERGER *et al.*, 1954b
Benign tumours	Human	Rectal polyp	Over 700	2–8	2–4	LEUCHTENBERGER, 1954; LEUCHTENBERGER *et al.*, 1956a
Malignant tumours	Human	Breast	Over 10,000	2–4	2–4	LEUCHTENBERGER, *et al.* 1954b; 1956a; LEUCHTENBERGER, 1954
		Prostate		2–8	2–4	
		Kidney		2–4	2–4	
		Urinary bladder		2–16	2–8	
		Testis		2–8	2–4	
		Cervix		2–8	—	
		Stomach		2–8	2–4	
		Intestines		2–8	2–4	
		Pancreas		2–16	2–8	
		Liver		2–16	2–16	
		Pleura		2–8	—	
		Lung		2–8	2–4	
		Lymph Node		2–16	2–4	
		Bronchus		2–8	2–4	
Tumours	Mouse	Sarcoma 180	Over 1000	2–8	—	LEUCHTENBERGER, 1950, 1956; LEUCHTENBERGER *et al.*, 1952
		Ehrlich Ascites		4–16	—	
		DBA Lymphoma		2–8	2–4	
		Polyoma:	Over 1000			LEUCHTENBERGER *et al.*, 1960 in press
		Kidney		2–8	2–4	
		Lung		2–8	2–4	
Tumours	Chicken	Rous Sarcoma	Over 100	2–8	2–8	LEUCHTENBERGER *et al.*, 1960 in press

TABLE 4. PATHOLOGICAL PROCESSES WITH DNA QUANTITIES FREQUENTLY IN EXCESS OF THOSE OF NORMAL RESTING HOMOLOGOUS CELLS AND NOT EXPLAINABLE ON THE BASIS OF MITOSIS, POLYPLOIDY OR POLYTENY

Pathological process	Species	Type of tissue or cell	Number of cells analysed	Range of DNA values in cells in basic units		References
				Abnormal	Normal	
Virus infections with verruca vulgaris	Human	Skin	Over 500	2–28	2	LEUCHTENBERGER, 1957b; LEUCHTENBERGER and LEUCHTENBERGER, 1957; LEUCHTENBERGER *et al.*, 1959
Adenovirus infection (Types 3, 7)	Human	Tissue culture HeLa cells Amnion cells	Over 750	 4–84 2–30	 4, 8 2, 4	LEUCHTENBERGER *et al.*, 1959; BOYER *et al.*, 1957, LEUCHTENBERGER and BOYER, 1957
Suspected virus infection	Human	Kidney Adrenal Seminal vesicle Lung	Over 600	2–45 2–16 4–34 2–18	2 2, 4 2, 4 2, 4	LEUCHTENBERGER, 1957b; LEUCHTENBERGER *et al.*, 1959; MORITZ and LEUCHTENBERGER, 1955
Focal hepatic changes (after injection with tumour DNA	Mouse CF_1 BALB/C	Liver	Over 1000	4–16 8–32	4 8	LEUCHTENBERGER *et al.*, 1958b

TABLE 5. PATHOLOGICAL PROCESSES WITH DNA QUANTITIES FREQUENTLY DEFICIENT IN RELATION TO NORMAL CELLS

Pathological process	Species	Type of tissue or cell	Number of cells analysed	Range of DNA values in cells in basic units		References
				Abnormal	Normal	
Male infertility	Human	Sperm	Over 20,000	Less than 1	1	LEUCHTENBERGER *et al.*, 1953, 1955, 1956 b and c; LEUCHTENBERGER, 1959; LEUCHTENBERGER and LEUCHTENBERGER, 1958; WEIR and LEUCHTENBERGER, 1957
		Spermatid		Less than 1	1	
		2° Spermatocyte		Less than 2	2	
		1° Spermatocyte		Less than 4	4	
	Cattle	Sperm	Over 12,000	Less than 1	1	LEUCHTENBERGER *et al.*, 1956 c and d; LEUCHTENBERGER, 1959; LEUCHTENBERGER and SCHRADER, 1955
		Spermatid		Less than 1	1	
		2° Spermatocyte		Less than 2	2	
		1° Spermatocyte		Less than 4	4	
	Clam	Sperm	Over 500	Less than 1	1	ITO and LEUCHTENBERGER, 1955
		Spermatid		Less than 1	1	
		2° Spermatocyte		Less than 2	2	
		1° Spermatocyte		Less than 4	4	
Hereditary pituitary dwarfism	Mouse	Liver	Over 600	2	2, 4, 8	LEUCHTENBERGER, *et al.*, 1954a
		Pancreas		2	2, 4	
Dwarfism	Cattle	Adrenal	Over 1600	Less than 2	2	LEUCHTENBERGER, 1959; LEUCHTENBERGER and SCHRADER, 1955
		Brain		Less than 2	2	
		Seminal vesicle epithelium		Less than 2	2	LEUCHTENBERGER *et al.*, 1956d
		Sperm		Less than 1	1	
		Spermatid		Less than 1	1	
		2° Spermatocyte		Less than 2	2	
		1° Spermatocyte		Less than 4	4	
Late stage pycnosis	Mouse	Liver	Over 200	Less than 2	2, 4, 8	LEUCHTENBERGER, 1950
		Sarcoma 180		Less than 2	4–8* (mitosis)	
Early irradiation effects	Rabbit	Bone marrow	Over 1000	Less than 4 Mostly 2	2–4 (mitosis)	UYEKI *et al.*, 1959

* Fresh viable tumour.

analysed and range of DNA values is given in Tables 2 to 5. It can be seen from Tables 1 to 5 that, in contrast to the constancy of DNA under normal physiological conditions, the DNA content of cells in pathological processes varies considerably, disclosing a wide spectrum of values. In some pathological processes, the DNA content remains unchanged (Table 2); in others it frequently increases (Tables 3 and 4), while in others it often decreases (Table 5).

This report will deal mainly with the quantitative behaviour of DNA related to four pathological processes, namely: surgical stress, tumours, virus infections and infertility. The pathological conditions were selected mainly for the following reasons: Each of them is an important, frequently-encountered disturbance, involving, however, quite different cellular systems and functions. Furthermore, there is a striking difference where their consequences for the organism as a whole are concerned. Surgical stress is more or less a reversible disturbance, usually without severe implications; tumours invading the normal tissues will lead to death; infection of cells with viruses may either induce temporary cell disturbances or also have serious consequences for the host, and infertility involves even the progeny.

1. SURGICAL STRESS

The influence of surgical stress on physiologic and metabolic processes of the organism is well known. The changes in the chemical composition of the body fluids, the electrolytes, in carbohydrate and protein metabolism, and hormonal activity of the adrenal and pituitary glands observed in the postoperative period are a few examples which serve to emphasize the alterations following surgical stress. While there can be no doubt that such alterations must be an expression of profound chemical cellular changes in the organs, very little work has been done in the past in a direct study on cells themselves. While this may seem surprising at first, it can probably be explained in part by the lack of suitable methods which would allow such chemical studies on a cellular level.

Attempts to study the problem on a cellular level were started in our laboratory by J. COLE and C. LEUCHTENBERGER (1956a, b; COLE *et al.*, 1958). Cells of liver and adrenals of dogs subjected to gastrectomy and cholecystectomy were analysed for their DNA and tyrosine content. Inasmuch as the adrenal gland is an organ intimately concerned with the body's response to stress, an investigation of the cytochemical behaviour of the adrenal after major surgery appeared to be particularly pertinent.

The use of microspectrophotometry is especially favourable for the study of the adrenal because the different zones of the adrenals can be analysed individually from cell to cell as to their chemical composition and their morphological appearance.

In analysing over 7000 individual cells from livers and adrenals from seventeen dogs, we found that the DNA content remained unchanged. The stability of the DNA content is the more remarkable since a marked elevation of tyrosine was noted in liver cells and in the cells of the adrenal medulla after surgery. This response, which was present in animals as early as 3 hr following surgery, assumes added significance when we bear in mind that this is being observed in the cells of

organs not directly involved by the surgical trauma. Of special interest is the finding that cells of the adrenal cortex revealed only slight changes in the tyrosine content as compared to the cells of the adrenal medulla. The differential response of the cells from the different parts of the adrenal after surgery is in keeping with the metabolic activities in the two portions of the gland.

As far as we know, tyrosine does not seem to play a role in the formation of cortical hormones and, therefore, it is not surprising to find only slight changes in the tyrosine content in the glomerulosa and fasciculata. On the other hand, the marked elevation of tyrosine in the cells of the adrenal medulla merits special consideration in view of the relationship between tyrosine and epinephrine. Recent biochemical evidence presented by UDENFRIEND and WYNGAARDEN (1956) has clearly shown that tyrosine is a precursor of epinephrine and norepinephrine. Furthermore, studies of patients who have undergone severe stress, such as burns, show increased excretion of epinephrine and norepinephrine. In our own experiments, the striking increase in tyrosine within the cells of the medulla after surgery may be looked upon also as a response of the adrenal to major stress at a cellular level. We are cognizant of the fact that data on epinephrine excretion and epinephrine synthesis are not wholly comparable; nevertheless, the suggestion may be made that the two phenomena are interrelated. Further support of this concept is found in the fact that the cellular tyrosine increase occurs as early as 1 to 6 hr after stress and the peak urinary excretion noted by BERKE *et al.* (1953) occurred within the first 8 hr following the burns.

The finding that DNA and tyrosine of the same cells respond quite differently when dogs are exposed to surgical stress is in accordance with results obtained in other species and other tissues involving different abnormalities. As can be seen from Tables 1 and 2, hypertrophy and hyperplasia of the prostate in the human and early bronchitis in mice after exposure to cigarette smoke similarly disclose a stability of the DNA content in spite of an increase of intranuclear and intracytoplasmic proteins (PERSKY and LEUCHTENBERGER, 1957; LEUCHTENBERGER *et al.*, 1958a). Such data certainly support the DNA-constancy theory of BOIVIN, VENDRELY and VENDRELY and are similar to DNA results in normal cells undergoing protein synthesis (SCHRADER and LEUCHTENBERGER, 1950). Nevertheless, one might have expected that protein synthesis, which is caused by an abnormal condition, would be reflected in the behaviour of the DNA. Perhaps the fact that these pathological processes are more or less self-limiting and even reversible, such as is the case for early bronchitis or surgical stress, may explain the unalteredness of the DNA. Furthermore, the fact that proteins are increased instead of decreased may also play a role in the preservation of the DNA quantity. A loss of proteins from cytoplasm and nucleus, such as was found to occur during pycnosis of cells (LEUCHTENBERGER, 1950), is associated with a progressive decrease of DNA, resulting in the death of the cell. (See Table 1[11], [12]).

2. TUMOURS

Ever since it was established that the nucleoproteins are the essential building-stones of cells (MIESCHER), tumours have been considered to contain larger amounts of DNA than normal cells. This concept was suggested by the increase in size and

stainability so frequently observed by pathologists in tumours. However, there were no chemical data to confirm or negate the concept. This may seem surprising, but it can be explained by the lack of adequate chemical methods for attacking such a problem. One must keep in mind that, in order to answer the pertinent question of whether or not the DNA content of a tumour cell is different from that of a normal cell, a special method is needed which permits the chemical analysis of a single cell. It is obvious that the conventional biochemical procedure, which determines the DNA content in a mashed neoplastic tissue, cannot give an answer, because it yields only a value which refers to the fresh or dry weight of the tissue without discrimination between cellular and non-cellular material. Even if biochemical analysis is done on a mass of isolated cells or nuclei (which, in most tumours, is nearly impossible because of the technical difficulties encountered in isolating nuclei of human tumours), the DNA content per cell is only an average value computed from the analysis of a mass of cells. While such an average value may be representative for single cells in cell suspensions with a uniform DNA content, it is not significant for the single cell, nor does it reveal any variation from cell to cell if the suspension analysed consists of cells with varying DNA content. The difficulties and pitfalls encountered in interpreting the biochemical results in suspensions of polyploid cells, even in normal tissues, such as the liver, have been demonstrated in earlier work by LEUCHTENBERGER *et al.* (1951).

However, with the availability of microspectrophotometry, the question concerning the relation of DNA to tumours became approachable. In view of the obvious importance of malignant transformation for the human, we began an extensive study on human tumours in our laboratory, using Feulgen-microspectrophotometry (LEUCHTENBERGER *et al.*, 1954b; LEUCHTENBERGER, 1954; LEUCHTENBERGER *et al.*, 1956a). Since very little was known about the DNA content of cells in normal human tissues prior to this study, an extensive comparative study on the DNA content of a variety of normal and malignant human tissues had to be carried out to establish a baseline. DNA measurements were made of nearly 7000 individual cells from seventy-one normal and fifty-two malignant tissues. It was found that all the normal tissues, no matter what their origin or metabolic function, contained cells with a similar basic mean DNA content, a finding which was in accordance with the original observations in animal tissues. This DNA content (2·8 arbitrary units, or $5{\cdot}6 \times 10^{-9}$ mg) was approximately twice that found in the sperm cells and is characteristic for human cells with a diploid chromosomal complement. Some adult tissues, such as the liver, also carry cells with nearly exact multiple amounts of the diploid cells, indicating correlation of the DNA content with the presence of multiple chromosomal complements, that is, polyploidy or polyteny.

In contrast to this constant and orderly pattern of DNA in normal tissues, the DNA content of precancerous lesions and malignant tumours was found to be considerably higher and revealed a much larger scatter from cell to cell. Characteristic examples are given in Fig. 1. It illustrates the difference in DNA behaviour between a primary adenocarcinoma of the stomach (Fig. 1a), a metastasis from this tumour to a lymph node (Fig. 1d), both disclosing high and scattered DNA values, and the surrounding normal portions of stomach (Fig. 1b) and

lymph-gland free of tumour (Fig. 1c), the latter disclosing constant diploid DNA values. Thus the DNA findings in tumours are indeed in accordance with the morphology of tumour cells exhibiting increase in size and staining intensity. However, since increase in DNA is also found in cells of rapidly-growing normal tissues undergoing mitosis, such as embryonic tissues, the deviating DNA values in malignant tissues are probably due to the mitotic process in tumours and cannot be considered a specific criterion for diagnosing malignancy *per se*. On the other hand, it must be kept in mind that normal adult tissues do not usually undergo mitosis and, consequently, exhibit the constant basic amount of DNA in their cells as shown for normal stomach and lymph node in Fig. 1.

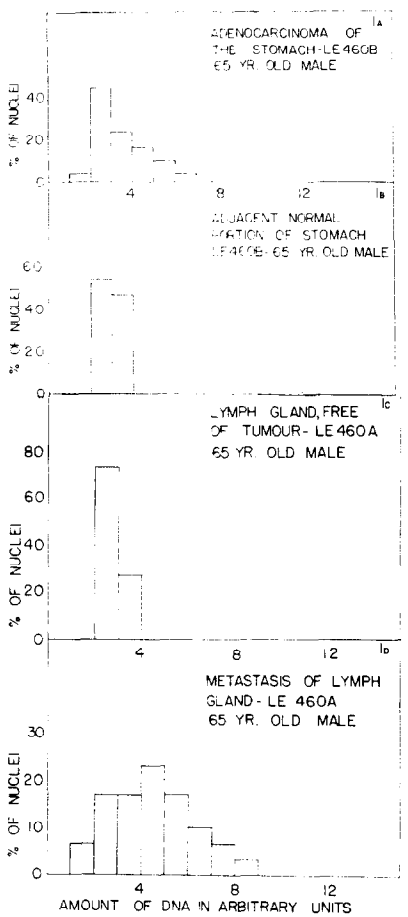

FIG. 1. Amount of DNA (microspectrophotometry) in human tissues

Therefore, an increase in DNA values in cells of an adult tissue should evoke suspicion of neoplastic growth unless regeneration is to be expected. One may argue, of course, that microscopic examination of a tissue for mitosis is much simpler and will lead to the same results. However, the absence of mitotic figures

in a tissue does not exclude the possibility that mitosis is taking place. If the time period of the mitotic cycle is very rapid, mitotic figures may be missed completely. Since DNA synthesis occurs at a very early stage of the mitotic process, namely, already in interphase, and is thus independent of the time period of the mitotic cycle, DNA increase can be used as a most sensitive indicator for a division process. Studies of this kind may be particularly helpful in the cases which the pathologist designates as borderline cases, such as carcinoma *in situ*, some cases of low-grade malignancy, and, in brief, cases in which mitotic figures are scanty or absent. Although these studies clearly reveal that the malignant process has no specific effect on the DNA content of a nucleus, nevertheless, deviating DNA values in cells which seem morphologically normal may indicate a preparation for abnormal growth and may, therefore, be of help in diagnosing early malignancy.

Furthermore, in some specific instances, DNA analysis may even permit separation between benign lesions and malignant ones occurring within the same organ. PERSKY and LEUCHTENBERGER (1957), who compared the DNA content in cells from twenty-two benign hypertrophic or hyperplastic prostatic glands with that in cells from twenty-five malignant prostatic glands, found a significant difference in the DNA behaviour. While the malignant cells showed the expected higher DNA values, the benign cells disclosed essentially a normal constant DNA quantity (Fig. 2). This difference in DNA behaviour deserves special consideration, since nuclear sizes as well as intracellular proteins were increased in both types of lesion, the benign and the malignant one (Fig. 2 and Table 1 (2)–(3)). The constant DNA values found in these benign conditions are in accordance with the cytological observations made in these same specimens. In spite of careful cytological examination and extensive search within these glands, mitotic figures were rarely encountered. Since there is this lack of mitosis associated with an absence of DNA doubling characteristic for formation of new cells, enlargement of the tissue must either have occurred previous to the surgery or be progressing at a very slow rate. It is felt that the integration of the cytochemical data with the clinical and histopathological findings is helpful in obtaining a better understanding of the true nature of these benign lesions. The suggestion that the benign lesions are the remains of a previous stimulus, with little residual growth activity, seems justified and is corroborated by their clinical course. Studies of this kind may be helpful when a definite diagnosis cannot be reached in consideration of the suspicious prostatic biopsy. When an area of DNA build-up is demonstrated in such a specimen, one would feel more secure in proceeding with radical surgery. A failure to demonstrate a build-up of DNA would suggest that, in terms of the growth potential, the tumour is of relatively low-grade activity, at least at the time of the biopsy.

In addition to the various types of human tumour studied by us, we also examined spontaneous and transplanted animal tumours for the relation of DNA to them (LEUCHTENBERGER, 1950, 1956; LEUCHTENBERGER *et al.*, 1952). Principally there were no essential differences in the DNA behaviour in human and in animal tumours studied. This similarity of DNA response deserves special consideration, because some of the animal tumours were tumours with a known viral aetiology, namely, Rous sarcoma of the chicken and Polyoma tumours of mice. The Polyoma

tumours, described first by STEWART (1955) seemed of particular interest, since injection of the virus into new-born mice will result in formation of different types of tumour, either sarcoma or carcinoma, and frequently in several organs of the same mouse. Furthermore, the sequence of events leading to these tumours can be studied with time, thus making it possible to relate changes in DNA content to gradual malignant transformations. One outstanding cytological feature during the development of this tumour is the early occurrence of peculiar, large "balloon-shaped" cells in tissues infected with the Polyoma virus.

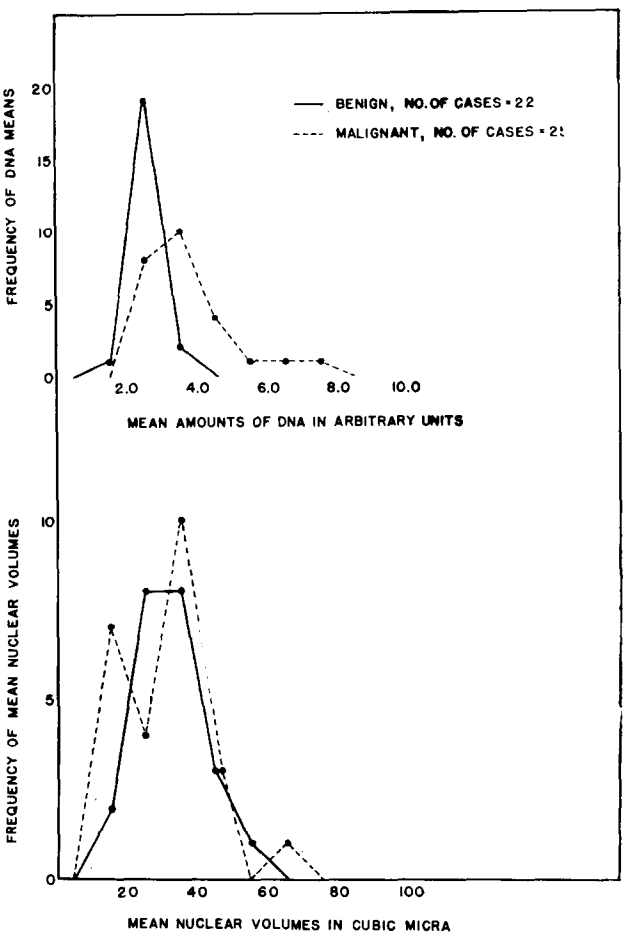

FIG. 2. Mean amounts of DNA in nuclei (Feulgen microspectrophotometry) and nuclear volumes of epithelial cells from benign hypertrophic, hyperplastic and malignant prostatic glands.

A characteristic example of the DNA patterns observed in normal kidney cells and during various stages of proliferation is given in Fig. 3. As can be seen, 10 days after infection of new-born mice, the DNA values for control- and Polyoma-infected mice are essentially the same, both disclosing values between two and four DNA, in accordance with the DNA synthesis during mitosis. However, starting with 17 days after infection, when the DNA values in the kidney of the controls are

predominantly two DNA, because mitosis is either absent or scanty, the kidneys of the virus-infected mice continue to show higher DNA values, in accordance with the presence of mitosis and abnormal proliferation. The similarity of DNA patterns observed in such virus tumours to that of other tumours is evident; in other words, here again the DNA increase can be explained just on the basis of mitosis, polyploidy or polyteny. The implications of this finding for host-cell–virus relationship

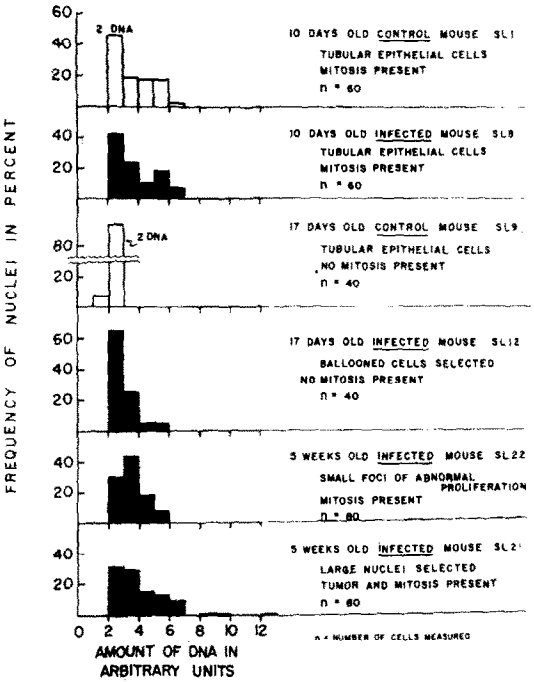

FIG. 3. Comparison between amounts of DNA* (Feulgen microspectrophotometry) in kidney cells from control mice infected at birth with polyoma virus.

in virus infections leading to proliferation of cells as compared to the host-cell–virus relationship in virus infections leading to death of cells will be discussed in the following section.

3. VIRUS INFECTIONS

In recent years analysis of many viruses has revealed that viruses contain chemical components similar to those of cells, namely DNA or RNA, or both. In view of the fundamental difference between location and function of DNA and RNA in normal cells, the study of the effect of DNA and RNA viruses on host-cells provoked our keen interest. Among the many problems concerning the peculiar relationship between virus and host-cells, we focused our special attention on the following question: Is there a cytological and cytochemical difference in the response of cells after an infection with a DNA virus as compared to that following an infection with an RNA virus? To study this question we first explored DNA-containing viruses, with particular emphasis in regard to their effect on the intranuclear DNA of the host-cell. Because of the importance of DNA as an essential chemical constituent of cells and its quantitative stability in normal cells, the study of DNA in

cells after they have become infected with a virus which itself contains DNA obviously assumes great interest when one is attempting to elucidate virus–host-cell relationship. Since the question arose as to whether the response of the intracellular DNA to the virus DNA is more or less a characteristic one, different types of cells infected with different DNA-containing viruses were studied and two main lines of approach were followed. The first line concerns the DNA behaviour of cells *in situ* of tissues from hosts with virus diseases; the second deals with the DNA behaviour of cells in tissue cultures infected with virus (LEUCHTENBERGER *et al.*, 1959).

One of the well-known virus diseases of the human skin is the common wart, verruca vulgaris. In contrast to the uninvolved epithelial cells, the infected skin cells show large nuclei filled with bizarrely shaped, often grapelike, masses of DNA-containing material, or crystal-like structures. In later stages a bursting of the

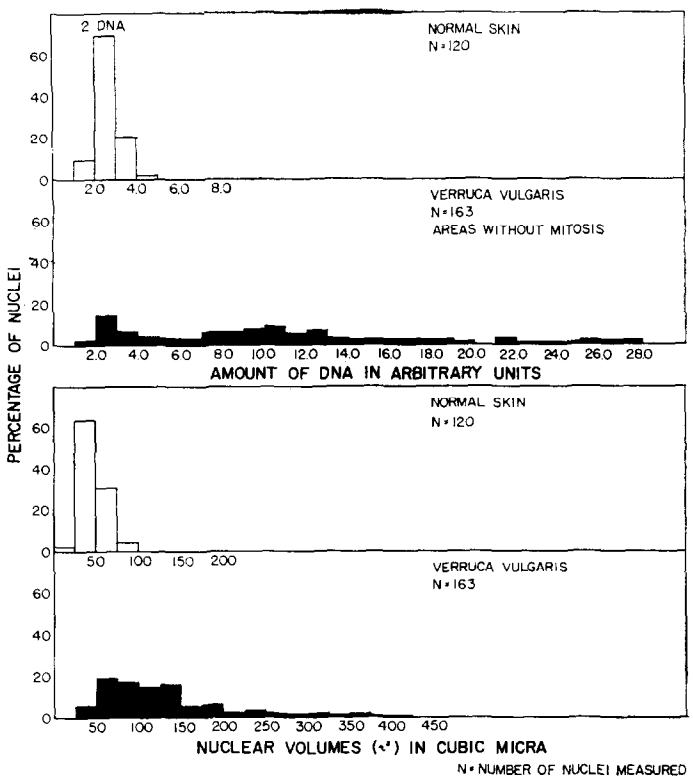

FIG. 4. Comparison between amounts of DNA (microspectrophotometry), nuclear sizes of individual cells from normal skin and skin infected with verruca vulgaris.

cells, releasing DNA material, and death of the cells themselves, are observed. When nuclear size and DNA content are determined in individual cells of normal skin and compared with those infected with verruca vulgaris, data are obtained of which a characteristic example is shown in Fig. 4. It is evident that the nuclear sizes and the DNA values (as obtained by Feulgen microspectrophotometry) of the normal skin

cells show a relatively narrow range; that is relatively little variation from cell to cell. The mean DNA value corresponds to the characteristic basic two DNA value found for diploid cells of all human tissues that we have examined thus far, and is twice that contained in the haploid human sperm. In contrast to the uniform size and stability of DNA in normal skin cells, the cells of the skin infected with verruca vulgaris exhibit not only larger and more variable nuclear sizes, but also strikingly higher DNA values, with considerable variability of the DNA content from cell to cell. It thus appears that infection with verruca vulgaris interferes with the stability of the DNA content of the normal skin cells and elicits production of abnormally high amounts of DNA within the nuclei. Since these increased quantities of DNA were found in areas of skin without mitosis, the high DNA amounts cannot be explained on the basis of an abnormally rapid cell division, as we reported to be the case in human tumours (LEUCHTENBERGER *et al.*, 1954b). It is also highly improbable that polyploidy or polyteny may be responsible for these high DNA values, because, instead of well-defined multiple groups of two DNA, four DNA and eight DNA such as are found, for example, in polyploid human liver, a more or less continuous increase of DNA is observed in nuclei after infection with verruca vulgaris. Furthermore, the cytological appearance of the chromatin in cells with polyploidy or polyteny formation is distinctly different from the abnormal configuration of the DNA material in the nuclei of the verruca vulgaris infected cells.

In order to determine whether this abnormal cytological and cytochemical DNA pattern is specific only for skin cells infected with verruca vulgaris in the human host, human cells of different tissues infected with different DNA-containing viruses, namely adenoviruses, were studied in tissue cultures. Tissue cultures are very favourable systems for such investigations, since the sequence of events in cells can be assessed from a cytological and cytochemical point of view at various known time intervals after the viral infection. Two types of cell were utilized: HeLa cells, a human cancer cell grown in tissue culture (BOYER *et al.*, 1957; LEUCHTENBERGER and BOYER, 1957) and first-generation tissue cultures of human amnion. The cytological response and the DNA pattern after infection with the same type of adenovirus were very similar in these two types of cell. Characteristic examples of the cytological and DNA changes observed in amniotic cells after infection with adenovirus type-7 are given in Fig. 5 and 6. The increase in nuclear size, the formation of bizarrely-shaped masses of DNA, the occurrence of DNA containing crystal-like structures, and the abnormally high DNA values are all features similar to those in verruca vulgaris. The absence of mitosis in cells which contain the large DNA masses is another important feature observed after infection with these DNA viruses. In spite of extensive search, we have been unable to find any indication of mitotic stages (including prophases); in other words, the cells infected with the DNA viruses seem to be unable to produce daughter-cells. The nuclei of the virus-infected cells become larger and larger, are filled with increasing DNA amounts, and finally burst—releasing DNA. When mitotic figures are observed in tissue cultures or in tissues *in vivo*, it can be easily recognized that the cells undergoing mitosis have not been infected by the virus. HARFORD *et al.* (1959), who studied HeLa cells infected with adenovirus, confirmed our light-microscopic studies by electronmicroscopy: no virus particles could be found in mitotic cells.

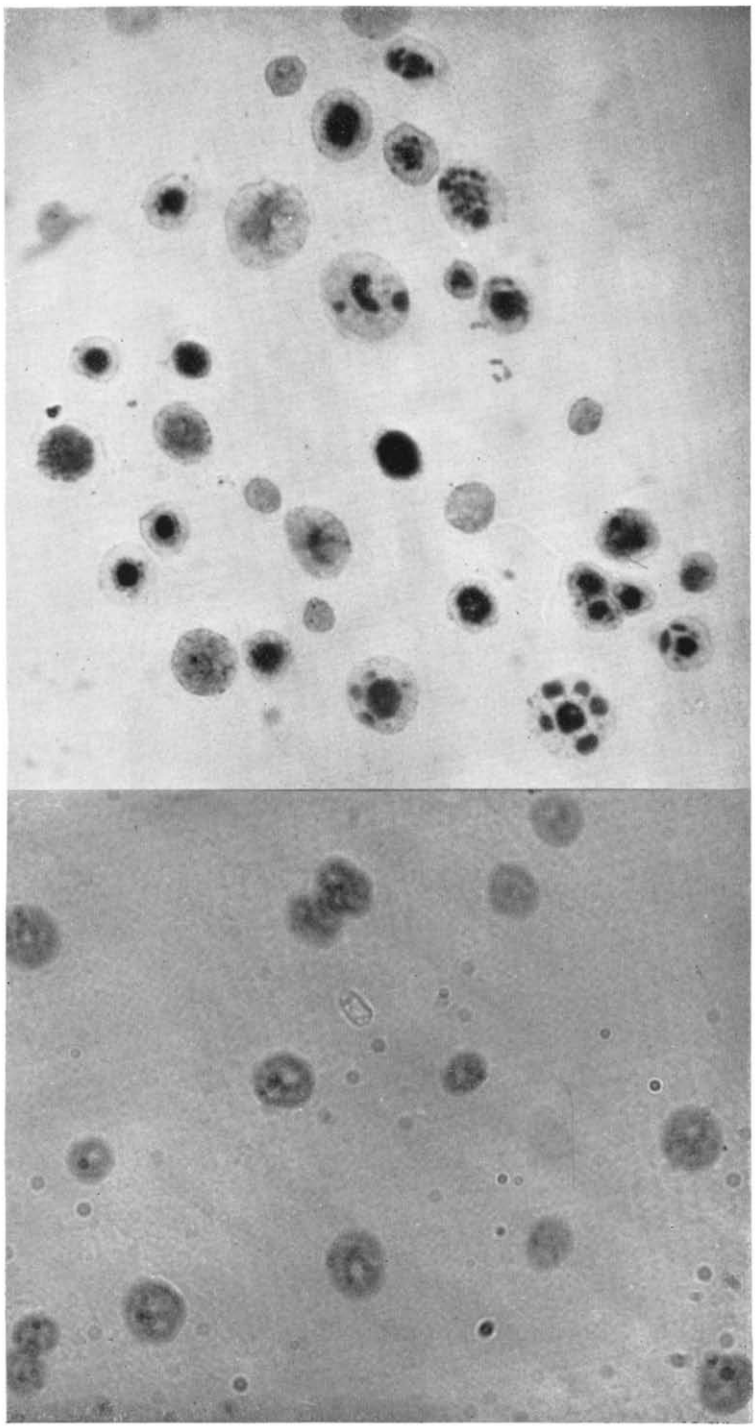

FIG. 5

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The cytological and cytochemical pattern of cells infected with these DNA viruses is in striking contrast to that of cells infected with an RNA virus, such as the Rous virus. Such cells show numerous mitoses leading to tumour formation and an increase in the DNA content explainable on the basis of DNA synthesis needed for the ensuing cell divisions. The same holds true for the tumours produced by the Polyoma virus. Although, at present, no information is available as to whether or not the Polyoma virus contains either RNA or DNA, our cytological and cytochemical studies suggest that we are dealing with an RNA virus having an effect on cells similar to that produced by the Rous virus (LEUCHTENBERGER *et al.*, in press).

The differential response of cells after an intranuclear infection with a DNA virus as contrasted with that following an infection with an RNA virus deserves special

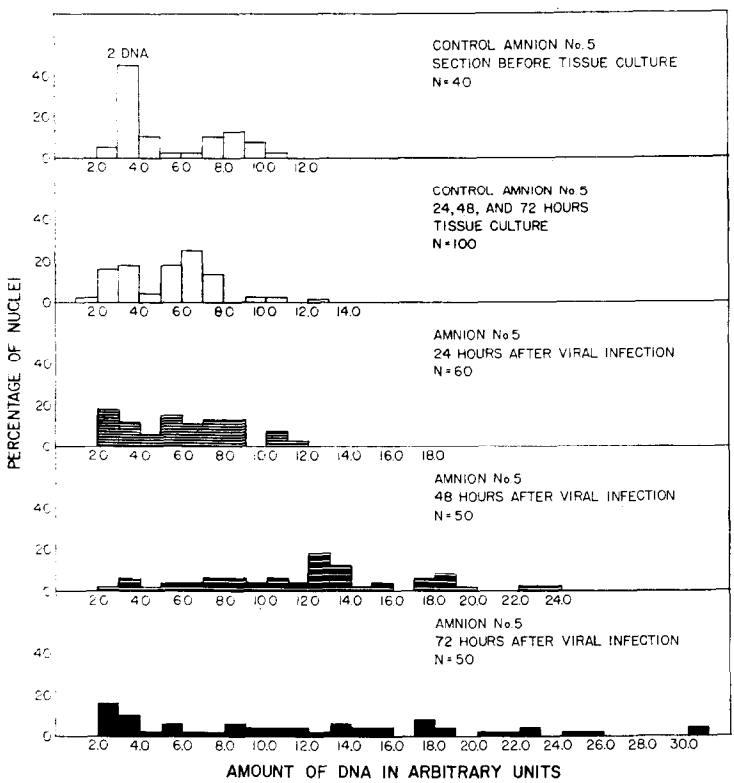

FIG. 6. Comparison between amounts of DNA (microspectrophotometry) in individual nuclei of human amniotic cells before and after infection with adenovirus type 7

comment, particularly in connexion with the problem of a possible viral origin of tumours. If our concept is correct that further propagation of daughter-cells is excluded whenever nuclei of cells are infected with DNA virus leading to these peculiar cytopathological and DNA changes, then it is highly improbable that an infection of cells with such a DNA virus can cause any tumours. It would seem much more likely that, if a virus is responsible for some tumours, it should be an

RNA virus causing proliferation of cells similar to that produced by the Rous virus.

The finding that intranuclear infection with DNA virus elicits an abnormal DNA metabolism in the nuclei leading ultimately to death of the cells can, at present, be interpreted only in a speculative way. One of the great difficulties, when attempting to explain the abnormally-high DNA values, comes from the impossibility of differentiating *in situ* between the intranuclear virus DNA and the host-cell DNA by the existing methods, including the Feulgen reaction. Therefore it cannot yet be decided whether the large DNA amounts observed are due to multiplying virus DNA or to an unusual and irregular synthesis of host-cell DNA

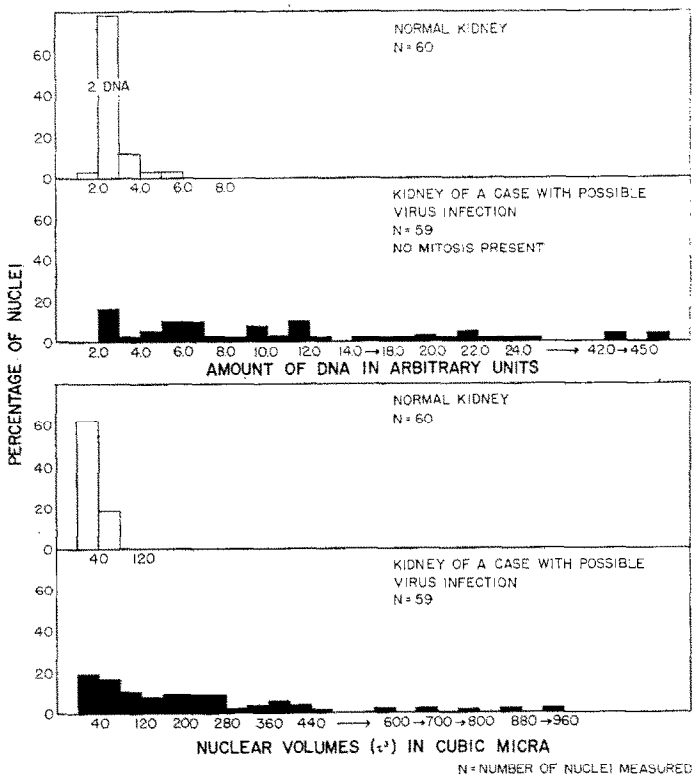

FIG. 7. Comparison between amounts of DNA (microspectrophotometry), nuclear sizes of individual cells from normal kidney and kidney from a patient with possible systemic virus disease.

induced by the presence of the virus. That the aberrations may reflect viral propagation is suggested by the similarity of the cytological and cytochemical behaviour of cells infected with other DNA viruses in which intranuclear viral synthesis occurs (YAMAFUJI *et al.*, 1954; LEUCHTENBERGER and BOYER, 1957). It thus appears that there is a uniform response of cells to an intranuclear infection with a DNA virus which seems to be almost specific for these virus-infected cells, irrespective of the type of DNA virus, irrespective of the type of cell infected (normal or cancer cell) and regardless of whether the cells are still *in situ* or have been explanted to grow in tissue culture. As a matter of fact, when the microscopist is confronted

with this peculiar combination of intranuclear bizarre configuration of DNA masses and increase and variability of DNA, which cannot be explained on the basis of mitosis, polyploidy or polyteny, he must seriously consider viral aetiology. Such a case, in a young man who died of an unexplained respiratory disease, was reported by MORITZ and LEUCHTENBERGER (1955). Nearly all the tissues examined contained cells with large nuclei and intranuclear alterations very similar to those found in the virus-infected cells described above. In Fig. 7 the nuclear size and the DNA values in the kidney of this man are graphed for comparison with those of a normal kidney. The similarity of the DNA pattern in the kidney nuclei of this case to those of the verruca- or adenovirus-infected nuclei is evident.

In this connection, some preliminary results which we obtained in our studies on the effect of tumour DNA on mice deserve to be mentioned (LEUCHTENBERGER *et al.*, 1958b). When white mice (BALB/C and CF_1) were injected intraperitoneally with DNA prepared from tumours of agouti C_3H mice, foci of unusually large atypical cells appeared in the livers. The cytological appearance resembled early changes observed in tissue cultures after infection with DNA viruses, such as formation of intranuclear eosinophilic inclusions. Furthermore, the increase in nuclear size was associated with increased and variable DNA quantities, in spite of conspicuous absence of mitosis. Injections of DNA prepared from spleens or livers of the same C_3H mice did not provoke a similar change in the livers.

The question of whether the effect of tumour DNA is due to an aberration in the DNA molecule or, perhaps, to a contamination with a virus not present in DNA of the normal organs must await further investigation. However, in view of the similarity between the findings obtained in cells with known viral aetiology, it is intriguing to speculate on the possibility that there may be a related cause for the cellular changes. Although we realize, of course, that proof of viral aetiology is lacking for the last two examples, the concept of a possible viral infestation is offered with the suggestion that other pathological tissue processes of unknown aetiology presenting similar findings would also be examined from a viral point of view.

4. INFERTILITY

One example where the relationship between an abnormal condition and the behaviour of DNA is of special interest concerns the problem of male infertility. It is well known that the analysis of sperm cells constitutes one of the fundamental criteria in studying the complex problem of fertility in the male. However, while it is generally recognized that volume and viscosity of seminal fluid, as well as counts, motility and morphology of the sperms, give basic information in the appraisal of fertility, there is no agreement concerning the decisiveness of any one of these factors in determining whether the sperms of a male are capable of normal fertilization. HOTCHKISS (1944) concludes that it is necessary to correlate all known data on the semen with the findings obtained by physical examination of the male to justify a division into fertile and sterile categories. The difficulties in gauging sperms for their fertilizing ability clearly indicate a lack in our knowledge of the detailed structure of the sperms, and suggest the presence of still unexplored elements which may play a conclusive role in the characterization of a sperm as fertile or sterile.

In view of the fact that the mature sperms carry mainly deoxynucleoproteins, we felt that an investigation of the possible role of DNA for the problem of male infertility might be of importance. Therefore, during the years 1951 through 1958, we undertook a very extensive study, examining by means of Feulgen microspectrophotometry over 32,000 individual sperm cells from 200 men and over 100 bulls for their DNA content (LEUCHTENBERGER *et al.*, 1953, 1955, 1956 b, c and e, LEUCHTENBERGER and LEUCHTENBERGER, 1958; LEUCHTENBERGER, 1959; WEIR and LEUCHTENBERGER, 1957). The seminal fluids of the human males were obtained through the Infertility Clinic of the Maternal Health Association in Cleveland. The fertile men were fathers of one to three children and the infertile men were the husbands of barren couples who had been evaluated according to the standards established by the American Society for the Study of Sterility (1951); the wife in each case was assumed to be fertile. The seminal fluids of the bulls were secured from breeding stations in Ohio, Virginia and Kansas. It should be emphasized that the DNA analyses were done only on

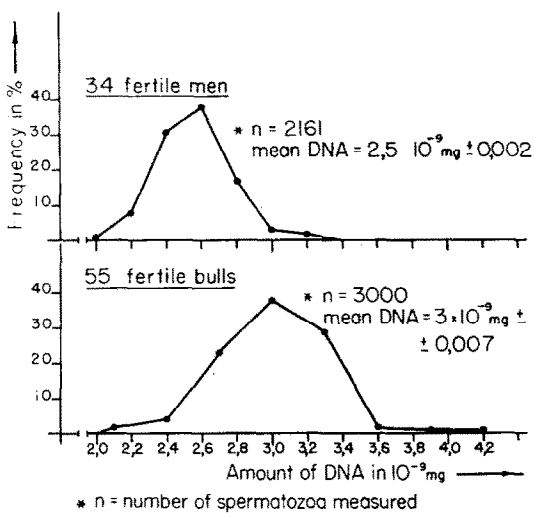

FIG. 8. Amount of DNA (Feulgen microspectrophotometry) in 5161 individual spermatozoa from 34 fertile men and 55 fertile bulls.

sperm cells with normal morphology and that, at the time when the DNA determinations were carried out in our laboratory, we had no knowledge as to whether the specimen examined came from a fertile or an infertile individual.

Since, prior to our studies, no systematic attempts had been made to determine quantitatively DNA in individual sperm cells of men and bulls, an extensive study of the DNA content in sperms from fertile males was carried out so that a baseline for comparison with infertiles could be established. Fig. 8 illustrates the results of Feulgen microspectrophotometric measurements of DNA in over 5000 individual sperms obtained in seminal fluids from thirty-four fertile men and fifty-five fertile bulls. Examining first the DNA data for the human sperms in the upper graph, it can be seen that there is very little variability in the DNA content from sperm to

sperm. Over 90 per cent of all sperms examined have a DNA content within 10 per cent of the mean DNA value of $2{\cdot}5 \times 10^{-9}$ mg. This striking constancy of the DNA content is also obtained in sperms of fertile bulls. The DNA values show a very narrow range, with little variability from sperm to sperm, and approximately 90 per cent of the sperms are within 10 per cent of the mean DNA values of 3×10^{-9} mg characteristic for bull sperm. In both humans and bulls,

Type of mammal and mean DNA	No of cases	Range of DNA
34 fertile men $2{,}5 \times 10^{-9}$ mg	7	2,3 - 2,39
	13	2,4 - 2,49
	12	2,5 - 2,59
	2	2,6 - 2,69
55 fertile bulls $3{,}0 \times 10^{-9}$ mg	5	2,4 - 2,79
	44	2,8 - 3,19
	6	3,2 - 3,59

FIG. 9. Range of amounts of mean DNA (10^{-9}mg) in spermatozoa from 34 fertile men and 55 fertile bulls.

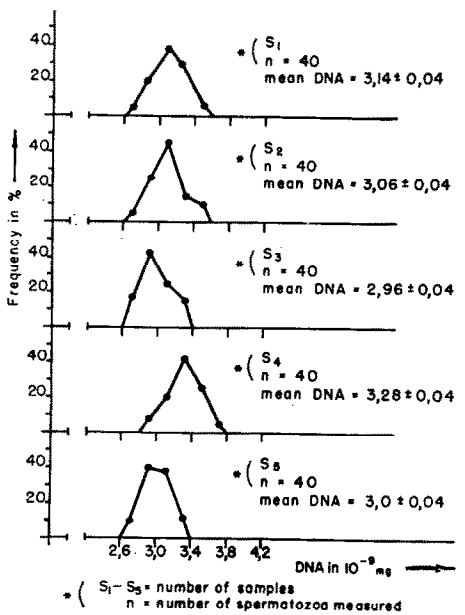

FIG. 10. DNA in individual spermatozoa of 5 different semen samples from the same fertile bull obtained at 2–3 months intervals.

the DNA content corresponds very well to the haploid chromosomal complement of 1*n*, which is characteristic for the sperm. The quantity of DNA in the haploid sperms is half of that found in diploid somatic cells of humans and cattle, respectively (LEUCHTENBERGER, 1959; LEUCHTENBERGER *et al.*, 1956d; LEUCHTENBERGER and SCHRADER, 1955). The DNA quantity in sperms is also very similar from man to man and from bull to bull within the fertile group, as can be seen from Fig. 9.

All thirty-four fertile men had a mean DNA content in their sperms which was very close to the value of $2{\cdot}5 \times 10^{-9}$ mg, the largest deviation being 15 per cent, and all fifty-five bulls had a mean DNA content close to 3×10^{-9} mg, the largest deviation being 20 per cent. Although these data strongly suggest that the DNA content in morphologically normal sperms is characteristic and extremely stable for each fertile individual, repeated analyses of different samples obtained over a long period of time from the same fertile males were carried out. Such DNA determinations revealed little variability in the quantity of DNA from specimen to specimen examined at monthly intervals, even within a period of 2 years. Fig. 10 gives an example illustrating DNA values for five repeat samples obtained from the same fertile bull at bimonthly intervals. It is evident that there is a similarity not only between the mean DNA but also between the distribution of the individual sperm DNA values from sample to sample. This constancy of the DNA content in sperms of various samples is the more remarkable since sperm counts and number of sperms with normal morphology frequently fluctuated in the same specimens. Fig. 11 shows a comparison between such data and the DNA content on twelve different samples obtained from the same fertile man within a period of 2 years. It can be seen that, in contrast to the constant DNA quantity in all samples, the sperm counts and the sperm morphology disclosed considerable variation from sample to sample.

Sample and date		Mean DNA in 10^{-9} mg	Sperm count in 10^6	Normal morphology in %
1	12/52	2,5	127	70
2	1/53	2,3	48	80
3	2/53	2,6	134	60
4	5/53	2,6	43	50
5	9/53	2,4	113	70
6	11/53	2,5	39	70
7	12/53	2,6	64	70
8	1/54	2,6	53	50
9	3/54	2,5	85	50
10	4/54	2,6	165	30
11	12/54	2,6	76	60
12	12/54	2,5	100	70

FIG. 11. Analyses of DNA content, counts and normal morphology of spermatozoa in 12 repeat samples from the same fertile male.

A further illustration of the DNA constancy and of the reproducibility of the DNA data obtained by Feulgen microspectrophotometry in sperms from fertile males is given in Fig. 12. It can be seen that the DNA values for individual sperms in seminal fluids obtained from three groups of fertile men at three different periods covering a number of years are indeed very similar, with each group having the characteristic haploid DNA content (expressed here in arbitrary units).

Turning now to the DNA data obtained in infertile males, one is justified in stating that the stability of the DNA content in sperms from fertile males represents an obviously good basis for comparison.

In contrast to the constant and uniform haploid DNA content carried in the sperms from fertile men and from fertile bulls, the DNA content from infertile men and infertile bulls is frequently significantly lower than in fertile and varies

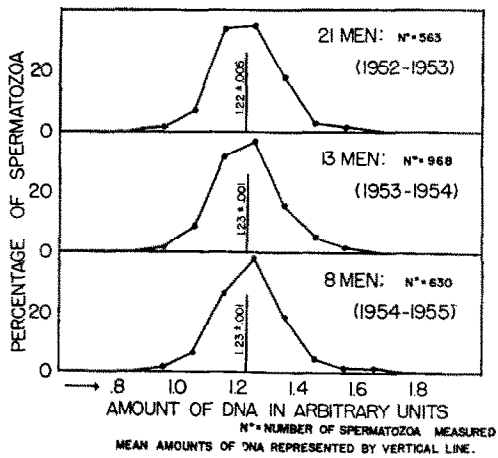

FIG. 12. Amount of DNA (microspectrophotometry of Feulgen reaction) in individual spermatozoa of fertile men. Studies done at different periods (1952–1955).

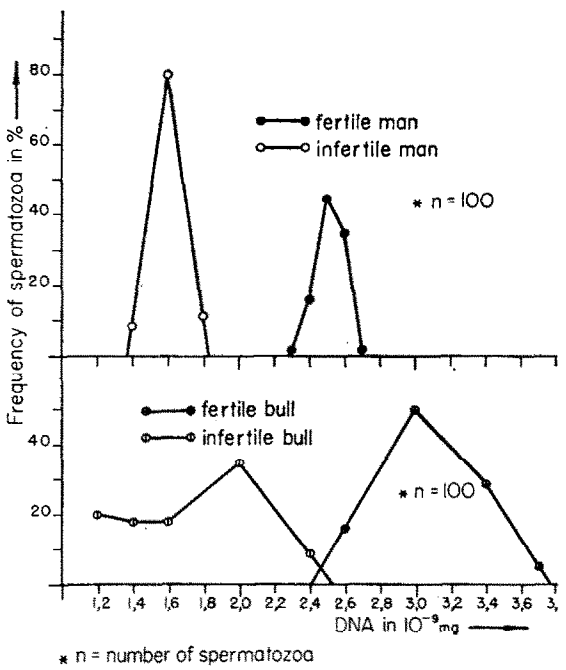

FIG. 13. Comparison of DNA content in individual spermatozoa between a fertile and an infertile man and a fertile and an infertile bull.

considerably from sperm to sperm, from sample to sample, and from individual to individual. Fig. 13 is an example illustrating the difference between the DNA content in sperms from a fertile and an infertile man and from a fertile and an infertile bull. It can be noted that the infertile man has significantly lower DNA values than the fertile man and that the infertile bull reveals not only a similar

DNA deficiency but also a larger scatter of the DNA content from sperm to sperm when compared with the DNA data obtained in the fertile bull.

This DNA deficiency in sperms of infertile males as contrasted to the normal haploid DNA content in sperms of fertile males is the more significant since, as pointed out previously, only sperms of normal appearance were selected for the DNA determinations. In other words, sperms with a normal DNA content from

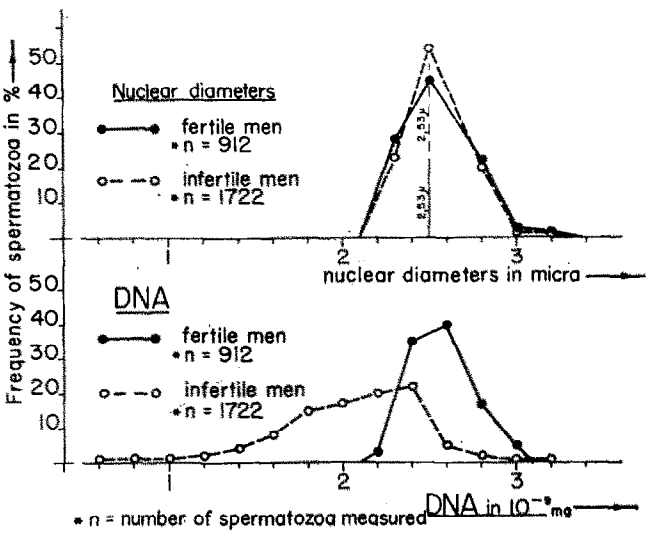

FIG. 14. Nuclear diameters and DNA content of sperm nuclei from fertile and infertile men.

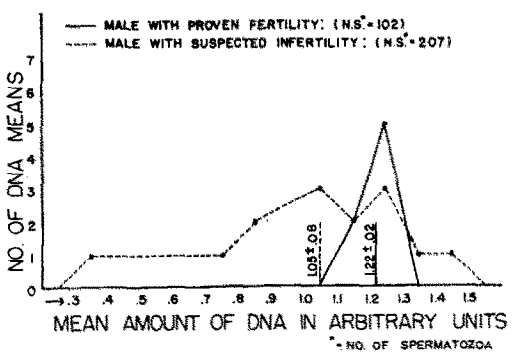

FIG. 15. Mean amount of DNA in spermatozoa of 7 individual semen samples of a male with proven fertility, and of 14 individual semen samples of a male with suspected infertility, taken at different intervals within a period of 17 months.

the fertile group and sperms with a deficient DNA content from the infertile group could not be distinguished cytologically. This statement is confirmed by data presented in Fig. 14. It can be seen that the sizes of sperm nuclei, in which the DNA was determined, are nearly identical for the fertile and infertile males, in spite of the fact that most of the sperm nuclei from the infertile group carried

significantly lower and more variable amounts of DNA than the sperm nuclei from the fertile group.

In view of the finding that each fertile male examined had always the same haploid DNA content in sperms in repeated samples, the question arose as to whether an infertile male with a low DNA content would similarly show persistent low DNA amounts in repeated samples. A study of fifty infertile males with at least four, and up to twenty, repeat samples for each infertile individual disclosed that a low DNA is by no means a permanent feature, but that the DNA amount in sperms may vary considerably from sample to sample for the same infertile male. Fig. 15 gives an example illustrating this variability. In contrast to the constant DNA amount present in all seven samples from the fertile male, the DNA values

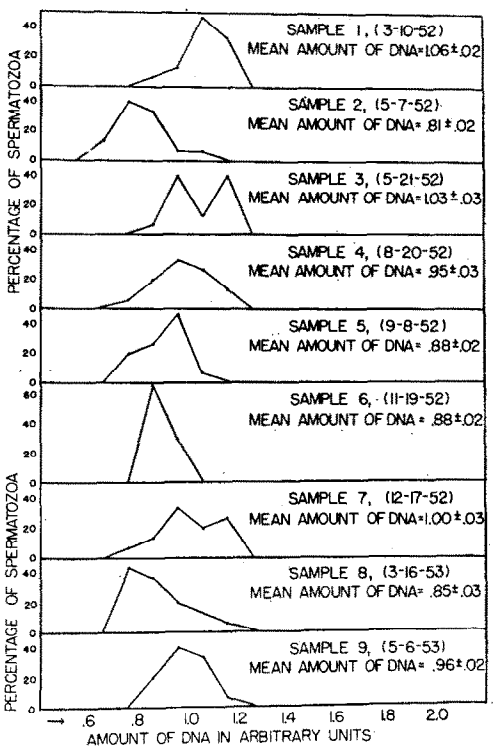

FIG. 16. Amount of DNA (microspectrophotometry) in 135 spermatozoa of the same infertile male (LE 212) at different intervals.

for the infertile vary from sample to sample, with some of the samples showing even a normal DNA value. In some infertile males, the DNA values were always abnormal for each sample; however, as can be seen from Fig. 16, the degree of DNA deficiency was not the same for each sample. The finding that a faulty DNA content is not necessarily a persistent feature and that it may not only fluctuate but even revert to a normal DNA in sperms of an infertile individual must be taken into consideration when trying to evaluate DNA data in sperms. A DNA analysis

of a single seminal specimen which gives a normal sperm DNA value is not indicative that the individual belongs to the fertile group. Only DNA examinations of repeated samples at various time intervals will reveal the DNA pattern and determine to which group the individual belongs. Of interest, however, is the fact that, in infertile males, the majority of repeat samples gave abnormal DNA values. For example, in a series of 108 specimens derived from eighteen infertile males, only 15 per cent of the samples had a normal mean DNA value, while the remaining 85 per cent were abnormal; whereas the counts, morphology and motility of sperms in the same specimens were all normal. As a matter of fact, in a series of thirty-five human couples with a mean duration of infertility of from 5 to 7 years or persistent infertility during the observation period, no significant defects in either man or wife could be detected, with the exception that thirty-three of the thirty-five males showed, at certain periods, abnormality of the DNA amounts in their sperms. Such data indeed suggest that a low DNA in sperms may possibly be one of the causes of otherwise unexplained infertility or prolonged duration of infertility. Although it is conceivable that a male with an occasional normal mean DNA value might be fertile at this period, this is by no means assured, but needs further study. Perhaps the frequency with which normal DNA values in sperms recur may be an important factor in gauging periods of infertility or complete sterility.

Because of the deficient DNA quantity carried in sperms of some infertile males, the question arises as to whether this deficiency is due to a loss of DNA from the

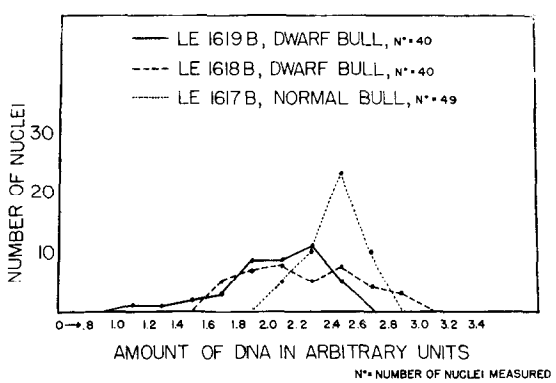

FIG. 17. Amount of DNA (microspectrophotometry) in spermatids of normal and dwarf bulls.

mature sperm *per se* or whether it can be traced back to testicular precursor cells, such as spermatogonia, primary and secondary spermatocytes, and spermatids. An investigation of this problem seemed of particular interest, because it would not only provide an answer to the question concerning at what stage during spermatogenesis the DNA disturbance occurs, but it would also permit the simultaneous exploration of histological, cytological and chromosomal behaviour in the testis and their relation to the DNA disturbance.

In pursuing this study, we examined over 2500 individual spermatogenic nuclei in testes from eight men and from eleven bulls for their DNA content. As fertile

human controls, the spermatogenic nuclei of testes were analysed from three men who were fathers of from one to two children and who died during the age period from 22 to 44 years. The infertile material consisted of testicular biopsies from husbands of barren marriages in which, according to the clinical investigation, neither husband nor wife showed significant defects which could be presumed to account for the barren marriage. As fertile bull control material, testes from fertile Horned Hereford bulls were analysed and compared with those of sterile Horned Hereford dwarf bulls or infertile Horned Hereford bulls suspected of carrying dwarf genes (LEUCHTENBERGER *et al.*, 1956d). DNA determinations in spermatogonia disclosed no significant difference between DNA amounts of fertile and infertile men and of fertile and infertile bulls. However, the DNA values of the primary spermatocytes, secondary spermatocytes and the spermatids of the infertile males were, in a number of cases, significantly lower than those of the fertile males. Fig. 17 illustrates a characteristic example of the difference between the DNA values in the spermatids of two sterile dwarf bulls and a normal fertile bull. In contrast to the narrow range of the DNA values of the spermatids of the fertile bull, the DNA values of the two dwarfs reveal not only significantly lower DNA values but also a much greater variability from spermatid to spermatid, the DNA behaviour being very similar to that found in the mature sperms. Of interest might be the finding that, although the DNA deficiency in these dwarfs could be traced back to the primary spermatocytes, neither the spermatogonia nor the somatic cells of other tissues revealed any DNA differences between dwarfs and normal bulls. Fig. 18 illustrates the DNA pattern in the livers of the same animals. It is evident that mean DNA content and distribution of DNA values from liver cell to liver cell are practically identical in the fertile and sterile dwarf bulls.

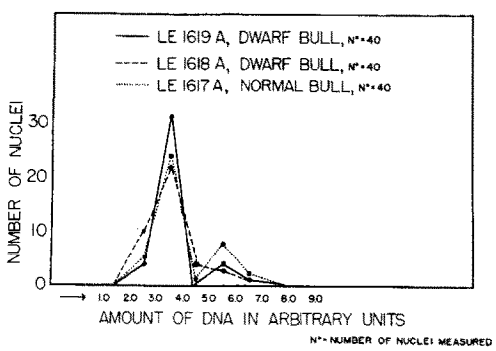

FIG. 18. Amount of DNA (microspectrophotometry) in liver of normal and dwarf bulls.

Considering now the DNA values of primary spermatocytes, secondary spermatocytes and spermatids, it should be stated that all the fertile men and fertile bulls examined showed very similar constant DNA values, with little variation from individual to individual. In contrast, the degree of DNA deficiency varied among infertile men from man to man, and, among infertile bulls, some bulls showed even normal DNA values in their spermatogenic cells.

However, in all cases examined, the primary spermatocytes revealed already the trend of the DNA pattern. When the DNA content of the primary spermatocytes was abnormal, then the DNA quantities in secondary spermatocytes, spermatids and sperms were abnormal, and, if the DNA in primary spermatocytes was normal, then the amount of DNA was also normal in secondary spermatocytes, spermatids and sperms. Fig. 19 illustrates the characteristic relationship between the DNA values of primary, secondary spermatocytes and spermatids in fertile men and fertile bulls and infertile men and bulls with DNA deficiency. It can be seen that the fertile man and the fertile bull have DNA values which are normal and in accordance with the chromosomal complement, giving a DNA ratio of 4:2:1 for

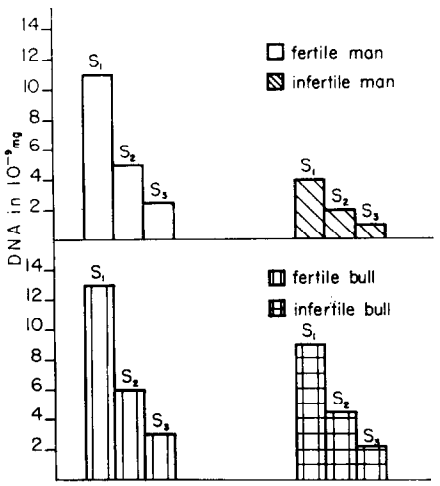

FIG. 19. Comparison of DNA content in primary (S_1), secondary spermatocytes (S_2) and spermatids (S_3) between a fertile and an infertile man and a fertile and an infertile bull.

primary, secondary spermatocytes and spermatids, respectively. In contrast, the infertile man, who, in repeat samples over a period of 2 years, always had deficient DNA amounts in his sperms, has significantly lower DNA values in all three types of spermatogenic cell. Similarly, the infertile bull reveals deficient DNA values in the primary spermatocytes, secondary spermatocytes and spermatids as compared with the DNA content in these cells from the fertile bull.

However, it can be noted that, in spite of the finding that the DNA deficiency is already present in the primary spermatocytes, a DNA ratio of 4:2:1 is maintained for the primary, secondary spermatocytes and spermatids. These results indicate that a DNA deficiency in the primary spermatocytes is apparently without any influence on the two following meiotic divisions, permitting the same precise halving of the deficient DNA amounts as is the case for primary spermatocytes which carry the normal DNA content. Indeed, the regular DNA ratios existing in the DNA-deficient germ cells are, in themselves, a plain indication that the loss of DNA did not occur in the mature cells, nor during meiosis, but that we are dealing with a deviation from the normal condition that exists already, before the germ cells have entered the meiotic period. In view of the importance attributed to

DNA for cell life, it seems somewhat surprising that a primary spermatocyte with a deficient amount of DNA should be able to divide in the same orderly fashion of 4:2:1 as a primary spermatocyte having the exact normal DNA content, even though, at each step, the DNA values are lower than those which characterize the corresponding ratios of germ cells from fertile males. This finding certainly suggests that formation of secondary spermatocytes, spermatids and sperms is not solely dependent on the exact DNA content of the precursor cells, but must be controlled by still unknown intra- or extra-cellular factors.

Whether deviation of DNA content in primary spermatocytes from infertile males is due to faulty DNA synthesis, to a loss of DNA from the chromosomes or to a loss of chromosomes cannot be answered with certainty at present, but needs further investigation. Although any of these disturbances or any combination of them may be responsible, the most ready explanation for the departure is to be sought in mitotic irregularities through which the distribution of the chromosomes is deranged. In any case, a cytological examination of the chromosomal conditions was indicated. Though we were aware of the difficulties involved, the considerable proportion of spermatogenic nuclei showing a very marked deviation from the normal amount of DNA in the infertile males led us to expect that, if chromosomal loss was responsible, this could be confirmed by chromosomal counts. It was not possible to obtain human material which was satisfactory for chromosomal counts; however, it was possible to get suitable preparations of bull testis, permitting combined DNA determinations and counting of chromosomes. Such studies revealed that the deficiency in DNA content apparent in so many primary spermatocyte nuclei of two sterile dwarf bulls was not paralleled by any demonstrable irregularity in chromosomal number, either at the prochromosomal stage which precedes or at the metaphase immediately following the stage at which the DNA determinations were done. The same number of chromosomes—sixty for the spermatogonial metaphase and at the meiotic prophase, and thirty at the first meiotic metaphase and anaphase—was counted for the fertile bull and for the two sterile dwarf bulls (LEUCHTENBERGER *et al.*, 1956d).

This unchanged chromosomal complement, which is present in spite of the DNA differences, is in good agreement with the finding that the cytological appearance of these cells, including their size, was essentially the same for the fertiles and infertiles. Admittedly, a larger number of infertile males should be examined as to their chromosomal complement. But, on the basis of the data now available, the variation and deficiency of the DNA values of infertiles cannot be ascribed to loss or irregular distribution of chromosomes. Actually, the regular DNA ratios of 4:2:1 observed in the primary spermatocytes, secondary spermatocytes and spermatids of the infertile males strongly suggest that the DNA deficiency is not a result of changes in the chromosomal complement but might rather be due to a disturbance involving a faulty DNA synthesis at the primary spermatocyte stage. Although the causes for the faulty DNA synthesis are unknown, there are indications that hormonal factors may, perhaps, play a role. As has been shown repeatedly, the process of spermatogenesis itself is under the influence of hormones, a finding which is compatible with our own observation that DNA deficiency is not a persistent feature in each infertile male, but that the degree of DNA deficiency may vary

from specimen to specimen and may even be normal in some samples. It is intriguing to speculate that perhaps a threshold quantity of hormones is required to guarantee the synthesis of a normal quantity of DNA in the primary spermatocytes and that, therefore, insufficient hormone production may be responsible for the faulty DNA synthesis. That a hormone deficiency may interfere with orderly DNA synthesis is indicated by our study on dwarf mice (LEUCHTENBERGER *et al.*, 1954a). Dwarf mice with a recessive hereditary anterior pituitary hypoplasia have only cells with the basic diploid amount in all somatic tissues, while their normal littermates have in certain tissues, such as liver cells, multiple DNA values in ratios of two DNA, four DNA, eight DNA. That lack of anterior pituitary growth hormone is responsible for the absence of DNA classes in these dwarf mice can be easily demonstrated. Treatment of such dwarfs with anterior pituitary growth hormone restores the multiple DNA classes, thus establishing a causal relationship between the hereditary anterior pituitary hypoplasia and the inhibition of the DNA classes in these mice. Although it is realized that the effect of the anterior pituitary growth hormone may apply only to DNA synthesis in somatic tissues, the observation that a hormone may exert an influence on DNA synthesis should stimulate further investigation in this direction. Systematic studies of the DNA content in spermatogenic cells of infertile bulls before and after treatment with pituitary growth hormone and other hormones, combined with controlled breeding experiments, may be of great interest and rewarding. If a relationship between DNA deficiency and certain hormones should be demonstrated, a fruitful avenue may be opened for combatting the problem of male infertility in cattle.

Whatever the causes of DNA deficiency may be, on the basis of the data presented, the capacity of a sperm to fertilize an egg seems to be closely linked to the exact haploid DNA content. That DNA deficiency in sperms may interfere with the sequential steps needed for proper fertilization has been shown by ITO and LEUCHTENBERGER (1955) in the clam, *Spisula solidissima*. This clam is an especially favourable model, because it permits the study of the fertilization of an egg by the sperm and DNA determinations before and after sperms have penetrated the eggs. As was the case with mammals, the clams could be separated into two groups: those which had the normal-DNA content and those which had deficient-DNA amounts in their spermatogenic cells. If eggs were exposed to sperms with the normal haploid DNA amount and to sperms with low-DNA quantity, a selective advantage for penetrating the egg could be observed for the sperm carrying the normal-DNA amount. Furthermore, all activated eggs contained sperms with the normal haploid DNA amount, while the great majority of the inactivated eggs revealed sperms carrying deficient amount of DNA. It therefore appears that DNA deficiency in sperms is harmful for the penetration and detrimental to the activation of an egg. Since penetration and activation are fundamental steps during fertilization, it is understandable that DNA deficiency in sperms may result in infertility.

Although we are aware of the fact that there may be many other causes leading to male infertility in mammals, it appears that DNA is of importance for the problem of fertility. It can hardly be considered a coincidence that all fertile males of two different mammals had a characteristic and constant DNA quantity, while infertile

males carried frequently lower and variable DNA quantities in their sperms. These findings strongly favour the concept that DNA deficiency in sperms may be responsible for at least one type of male infertility. One is also justified in saying that DNA analysis is helpful when gauging male infertility, particularly in diagnosing those cases in which the infertility cannot be accounted for by other defects.

This assessment of a few results may suffice to demonstrate the relation of DNA to various pathological conditions. It is evident that the DNA response to different pathological processes is quite variable; DNA may remain unchanged, or may be increased or decreased in quantity.

It is hoped that this report will stimulate the application of correlated histological, cytological and quantitative cytochemical techniques for the investigation of other intracellular substances and also further the studies of the many unexplored problems in pathology. The possibility of detecting chemical abnormalities in cells in spite of normal morphology, and of analysing simultaneously the sequential morphological and chemical changes in cells and tissues should advance our knowledge and understanding of pathological processes. It is felt that major advances in elucidation of disease will come from a close integration of such quantitative cytochemical and morphological studies.

This report is dedicated to Professor A. R. MORITZ, Director of the Institute of Pathology, Western Reserve University, whose vision and continuous help made possible the development of a cytochemistry laboratory at the Institute of Pathology.

SUMMARY

In view of the important role of DNA for cell life and cell continuity, its quantitative stability under normal conditions and its close relationship to the genes, we focused our special attention on the study of DNA in abnormal cells and in cells under pathological conditions.

During the last 10 years we have extensively investigated the quantitative behaviour of DNA in cells by Feulgen microspectrophotometry in a variety of pathological conditions, and significant information has been gained. We have also attempted to study in some of these processes other intracellular constituents, such as proteins, using particularly Millon microspectrophotometry and interference microscopy.

This report deals predominantly with the quantitative behaviour of DNA related to four pathological processes, namely: (1) surgical stress, (2) tumours, (3) virus infections and (4) infertility. These pathological conditions were selected mainly for the following reasons: Each of them is an important, frequently-encountered disturbance, involving, however, quite different cellular systems and functions, and each of them disclosing a different DNA behaviour. Furthermore, each exhibits a striking difference concerning the consequences of each process for the organism as a whole. Surgical stress is more or less a reversible disturbance, mostly without severe implications; tumours invading normal tissues may lead to death of the host; infection of cells with viruses may either induce temporary cell disturbances or may also have serious consequences for the host, and infertility involves the progeny.

(1) In an attempt to study the problem of surgical stress on a cellular level, dogs were subjected to gastrectomy and cholecystectomy, and cells of liver and adrenals were analysed for their DNA content. Inasmuch as the adrenal gland is an organ intimately concerned with the body's response to stress, an investigation of the cytochemical behaviour of the adrenal after major surgery appeared to be particularly pertinent.

The use of microspectrophotometry is especially favourable for the study of the adrenal because the different zones of the adrenals can be analysed individually from cell to cell as to their chemical composition and their morphological appearance.

Analysing over 7000 individual cells from livers and adrenals from seventeen dogs, it was found that the DNA content remained unchanged. This stability of the DNA content is the more remarkable since a considerable elevation of tyrosine was noted in liver cells and in the cells of the adrenal medulla after surgery. The finding that DNA and tyrosine of the same cells respond quite differently when dogs are exposed to surgical stesss is in accordance with results obtained in other species and other tissues involving different abnormalities.

(2) In view of the obvious importance of malignant transformation for the human, we started an extensive study on human tumours in our laboratory, using Feulgen microspectrophotometry. Since very little was known about the DNA content of cells in normal human tissues prior to this study, a comparative extensive study on the DNA content of a variety of normal and malignant human tissues had to be carried out to establish a baseline. DNA measurements were made of nearly 10,000 individual cells from seventy-one normal and fifty-two malignant tissues.

It was found that all normal tissues, no matter what their origin or metabolic function, contained cells with a similar basic mean DNA content, a finding which was in accordance with the original observations in animal tissues. In contrast to this constant and orderly pattern of DNA in normal tissues, the DNA content of precancerous lesions and malignant tumours was found to be increased and to reveal a much larger scatter from cell to cell. However, the deviating DNA data cannot be considered a specific criterion for malignant transformation of cells, but may be explained mainly on the basis of growth and mitotic processes present in most tumours. On the other hand, since most normal tissues in adult humans do not exhibit mitosis and, therefore, show a DNA constancy, an increase and large scatter of DNA in such a tissue must be looked upon with suspicion in regard to malignancy, unless regeneration is to be expected. Furthermore, in some specific instances, DNA analysis may even permit separation between benign lesions and malignant ones occurring within the same organ. For example, when we compared the DNA content in cells from twenty-two benign hypertrophic or hyperplastic prostatic glands with that in cells from twenty-five malignant prostatic glands, we found a significant difference in the DNA behaviour. While the malignant cells showed the expected higher DNA values, the benign cells disclosed essentially a normal constant DNA quantity. This difference in DNA behaviour deserves special consideration, since nuclear sizes as well as intracellular proteins were increased in both types of lesion, the benign and the malignant. The constant DNA values found in these benign conditions are in accordance with the cytological observations

made in these same specimens. In spite of careful cytological examination and extensive search within these glands, mitotic figures were rarely encountered.

In addition to the various types of human tumours studied by us, we also examined the relation of DNA to spontaneous and transplanted animal tumours. Principally there were no essential differences between the DNA behaviour in human and in animal tumours studied by us. This similarity of DNA response deserves special consideration, because some of the animal tumours studied were tumours with a known viral aetiology, namely Rous sarcoma of the chicken and Polyoma tumours of mice.

(3) In recent years analyses of many viruses have revealed that viruses contain DNA, RNA or both. In view of the quantitative stability of DNA in normal cells, the study of DNA in cells after they have become infected with a virus which itself contains DNA is obviously of great interest when attempting to elucidate virus–host-cell relationship. Since the question arose whether the response of the intracellular DNA to the virus DNA is a more or less characteristic one, we studied different types of cells infected with different DNA-containing viruses *in situ* in the host and in tissue cultures. Such infected cells revealed a peculiar combination of intranuclear bizarre configuration of DNA masses associated with striking increase and variability of DNA. This almost specific response of cells to an infection with a DNA virus was noted irrespective of the type of DNA virus (verrucca vulgaris, adenovirus, etc.), irrespective of species or types of cells infected (human, insect, normal or cancer cell) and regardless of whether the cells were still *in situ* in the host or explanted to grow in tissue culture. Since the virus-infected cells did not show any mitosis, the large DNA quantities cannot be explained on the basis of an abnormally rapid cell division such as is the case in tumours.

Although it cannot be decided at present whether the large DNA quantities are due to multiplying virus DNA or to an abnormal synthesis of host-cell DNA, or possibly to both, the changes rather suggest propagation of virus.

Of particular interest is the finding that none of the RNA-containing viruses which we have studied so far (Polyoma virus, Rous virus) has a similar effect on cells.

(4) One abnormality in which DNA studies by Feulgen microspectrophotometry have proved to be of special diagnostic value concerns the problem of male infertility. In order to investigate the possible role of DNA for the problem of infertility, over 32,000 individual sperm cells from 200 men and from over 100 bulls were analysed. In contrast to the remarkably constant and uniform haploid DNA content found in the spermatozoa of fertile males (both human and bulls), the DNA content in the spermatozoa from infertile males is frequently variable and significantly lower than that from the fertile ones. This finding is especially pertinent since the cytological appearance and the dry weight of the spermatozoa containing the normal and deficient amounts of DNA are identical. The same holds true for the spermatogenic cells; primary and secondary spermatocytes and spermatids each have a deficient DNA content in infertile males, as compared with the four DNA, two DNA and one DNA found in the fertile males, although the histological and cytological features of the testes and germ cells may be completely

normal. It thus appears that the DNA deficiency found by Feulgen microspectrophotometry in spermatogenic cells of normal cytological appearance can be considered as a criterion for gauging at least one type of male infertility and may have a bearing on the fertilization process.

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