Papers and Originals

Tissue-culture Approach to the Treatment of Cancer*

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[WITH SPECIAL PLATE]

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The present research began five years ago from the need for a means of distinguishing between the hormone-dependent and the autonomous types of human breast cancer. Such a method would be of value to the surgeon treating this condition, and would enable a more rational approach to be made to the disease, since only 50% of human breast cancers are oestrogen-dependent (Taylor, 1956) and at present there is no means of detecting these cases. A woman with metastatic breast cancer has therefore a 50% chance of being subjected to an unnecessary ablative operation such as hypophysectomy. The importance of recognizing the hormone-dependent type of mammary cancer has been emphasized by Hedley Atkins (Atkins *et al.*, 1960, 1964), whose group is investigating the problem by analysing the urine of women with the disease for 17-hydroxycorticosteroids and aetiocholanolone and attempting to predict the response to hypophysectomy or adrenalectomy from the combined pre-operative urinary levels of these steroids—the so-called “discriminant function” of Bulbrook *et al.* (1960). However, urinary steroid determinations have previously proved unreliable as a means of assessing hormone-dependence in this disease (Hobkirk and Forrest, 1957; Plantin *et al.*, 1958), and the physiological significance of such assays is still obscure (Bulbrook *et al.*, 1960). The predictive value of urinary steroid levels in the individual patient remains based upon statistical probability.

The present work represents a more direct approach to the problem. Since the pioneering work of Fell and Robison in 1929 it has been possible to keep organs from small animals alive outside the body for varying lengths of time on different types of media. It is only within the past 10 years, however, that these culture media have become sufficiently refined in chemical composition to enable cell metabolism to be studied by biochemical analysis of the medium on which the cells are maintained.

Glucose Utilization

Fig. 1 illustrates the utilization of glucose by the mammalian cell. In the oxidation of glucose pyruvic acid is formed as an intermediate. The fate of the pyruvic acid is governed by the current metabolic state of the cell. If the cell is deriving its energy from respiration via the Krebs cycle, then most of the pyruvic acid will be converted to CO_2 and H_2O . If, on the other hand, the cell is deriving its energy from glycolysis, then lactic acid will be the chief end-product of glucose metabolism.

It can be seen that if the cell is functioning in the complete absence of oxygen two molecules of lactic acid would be formed from each molecule of glucose used. The ratio of molecules of lactic acid formed to molecules of glucose used would therefore be 2—that is, L.A./G.=2. However, neither of these pathways, the glycolytic or the respiratory, operates to the complete exclusion of the other in the cell. It follows, therefore, that the theoretical value of two molecules of lactic acid produced from each molecule of glucose used is never attained under normal circumstances. The value of the L.A./G. ratio can be taken to indicate the functional state of the cell's enzyme systems. A ratio in the region of 1 indicates a fairly oxidative type of metabolism, in which 50% of the glucose is being converted to CO_2 . Such a ratio is obtained with thyroid gland cells for example. A higher ratio (approaching 2) means the cell is drawing its energy chiefly from glycolysis, as in the case of most cancer cells. These considerations assume that all the CO_2 is being formed from glucose; no account is taken of CO_2 derived from other sources—for example, that originating from protein intermediates entering the Krebs cycle (cf. Danes and Paul, 1961). Such other minor sources of CO_2 can be neglected, however, if the CO_2 being studied is radioactive and derived from radioactive glucose. By measuring such radioactive CO_2 , as well as lactic acid, produced from glucose during metabolism, the carbohydrate metabolism of the cell can be followed fairly accurately.

FIG. 1.—The oxidation of glucose.

It is known from the extensive studies of Vilee and his associates (Vilee and Hagerman, 1953; Gordon and Vilee, 1955; Vilee, 1962) and of Talalay and Williams-Ashman (1958) that one mode of action of oestrogens is to channel the glucose molecule towards more complete oxidation without altering the total amount metabolized—that is, for a given uptake of glucose by the cell, oestrogens increase the amount of CO_2 produced and therefore lower the L.A./G. ratio.

Organ Culture Technique

We decided to culture breast cancers outside the body and to assess their state of metabolism and their response to hor-

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mones by analysis of the medium for glucose and lactic acid and by the collection of carbon dioxide produced by the cells during respiration. We hoped that the hormone-dependence or otherwise of the cells would be reflected in a specific pattern of these measurements.

The method chosen to maintain the breast tissue outside the body was the organ culture technique of Trowell (1959). In this method the tissue under study is cut into a number of small slices approximately 2 by 2 by 2 mm. in size, and these are then placed on a metal grid in a Petri dish inside a special metal culture chamber (Fig. 2). The tissue lies in a gas phase

FIG. 2.—Trowell organ culture chamber (Type II). ACU, aluminium culture unit; GI, gas inlet; D, Petri dish; MG, metal grid with cultures; SRR, silicone rubber ring; GL, plate-glass lid.

and is nourished from below by medium in the dish. The medium and the gas are renewed every 48 hours. Trowell has shown that slices of many organs of the rat survive over a 10-day culture period under these conditions, and Hagmüller and Leslie (1962) were able to demonstrate a metabolic response to thyrotrophic hormone in human thyroid slices in the system. The method therefore seemed a suitable one to employ for the present work.

The culture unit was modified by the use of a stainless-steel lid with a funnel situated off-centre and closed by a self-sealing rubber cap. This permitted the injection of acid to release “bound” radioactive CO₂ from the medium. At the end of the culture period the total radioactive CO₂ produced by the cells from radioactive glucose in the medium was collected from the culture unit into an ionization chamber and its activity assayed on a Nuclear Chicago Corp. Dynacon 6,000 electrometer (Dickson and Leslie, 1963a).

When equivalent amounts of human breast tissue were maintained in each of several culture chambers in this way the results were extremely disappointing. Between replicate cultures there was no consistency of glucose uptake from the medium or lactic acid production into the medium or of CO₂ produced by the cells during respiration (Table I). The same

lack of uniformity was found in cultures of lymph nodes from patients with metastatic breast cancer. Inconsistent results were also obtained with normal tissues such as kidney and liver from guinea-pigs. The results indicated a deterioration of the cells over the culture period, and we were unable to prevent this despite many modifications of the technique. We were thus unable to establish a reliable baseline from the metabolic point of view on which to assess a hormone response in the breast tissue.

TABLE I.—Organ Culture—Human Breast Slices

Time (Days)	No. of Cultures	Tissue	Glucose Uptake* (μM/ml.)	LA/G	Percentage ¹⁴ C-G(U)→CO ₂ †
2	24	N	3.2–8.3	1.4–2.3	0.6–3.6
	24	NG	5.0–12.4	1.2–1.9	1.2–4.0
4	18	N	1.8–5.6	1.6–2.7	1.0–1.9
	18	NG	3.0–8.4	1.8–3.5	0.7–2.4
6	12	N	1.5–2.7	1.8–4.0	0.7–1.8
	12	NG	0.6–4.2	1.9–3.4	0.2–1.4

* Glucose at an initial concentration of 25 μM/ml. and activity of ¹⁴C-glucose was 0.5 μC/ml.

† Values are the percentages of metabolized ¹⁴C-glucose which were converted to ¹⁴CO₂.

N = Normal (control) tissue. NG = Malignant tissue.

The main reasons why we now believe this culture technique to be unsuitable for metabolic work are that the slicing procedure produces a variable and unpredictable degree of trauma to the cells, and, secondly, a tumour slice is in itself a variable—only 50% of the cells in the slice may be malignant, and of these an unknown number may be necrotic. It therefore became obvious that equivalent weights of tissue in the form of slices did not represent equal numbers of viable cells for culture. After two years of exhaustive investigation the tissue-slice technique was therefore abandoned and a new approach to the problem devised to overcome these difficulties.

Filter-well Technique

This was the filter-well technique (Fig. 3), and it consists essentially in disaggregating the tissue under study into a unicellular suspension by the use of enzymes such as trypsin. The cells in the suspension are then assessed for viability by means of a nigrosin dye-uptake test (Kaltenbach *et al.*, 1958) and counted on a haemocytometer. A known number of viable cells ranging from 10 million to 100 million is then layered on to a Millipore filter cemented to a Pyrex glass ring to form the filter-well.

A Millipore filter (Millipore Filter Corp., Bedford, Mass.) is a thin membrane of cellulose acetate containing millions of capillary pores of uniform dimension. The pores are direct channels through the filter and occupy approximately 80% of the total filter volume. Several different pore diameters are available, the filters used in the present work having a pore diameter of 0.65 micron. This is much smaller than the diameter of most mammalian cells, and thus the cells are retained on the membrane and at the same time there is free exchange of medium across the filter. The well, consisting of the Pyrex glass ring and its attached Millipore filter with the cells, stands on three glass legs in the Petri dish (Fig. 3). The cells lie in a gas phase as before. Thus in this technique the tissue slice is replaced by a population of cells of known number and viability.

The conditions for obtaining consistent and reproducible results with the filter-well were first established with cells of a human liver line (HLM) and with cells from freshly dissociated adult guinea-pig kidney. It was found that with equal numbers of cells in each well the cells would survive for 10 days or longer, and show during this time a consistent and meaningful pattern of metabolism in the form of glucose uptake and lactic acid and carbon dioxide production (Table II) (Dickson and Leslie, 1963a).

An unexpected finding with freshly dissociated tissues in this system was that the cells reaggregated on the Millipore filter to form organized histological structures. This can be strikingly demonstrated with cells from adult guinea-pig kidney, and Fig. 4 (Special Plate) illustrates the reorganization as it occurred in a population of 37 million adult guinea-pig kidney cells over a 10-day culture period.

After disaggregation by 1% trypsin (Difco Laboratories, Detroit, 1:250), and introduction into the filter-well as a unicellular suspension, the cells settle on the Millipore membrane after four hours to form a uniform evenly dispersed plaque (Fig. 4 A). On section this plaque shows a random arrangement of the cells with no structural features (Fig. 4 E).

At four days the cells were seen to have migrated centripetally to form a well-defined annulus (Fig. 4 B), which on section shows the beginnings of organization (Fig. 4 F). With time the annular colony continues to move centrally and the histology of the cells becomes correspondingly more complex. Fig. 4 C and G illustrate the situation at six days. By 10 days a well-defined central nodule has been formed (Fig. 4 D). On section this nodule shows a highly organized architecture with distinctive histological features (Fig. 4 H).

FIG. 3.—Trowell unit modified for filter-well cultures. ACU, aluminium culture unit; GI, gas inlet; HN, hypodermic needle inserted for liberation of CO₂ in medium; PD, Petri dish; FW, filter-well; RC, rubber cap; SRR, silicone rubber ring; SSL, stainless-steel lid.

TABLE II.—Metabolism of HLM Cells in Filter-Wells

Time (Days)	No. of Cultures	Glucose Uptake* (μM/ml.)	LA/G	Percentage ¹⁴ C-G(U)→CO ₂ *
2	24	14.5–15.6	1.58–1.73	—
4	24	14.0–15.7	1.60–1.65	1.0–1.5
6	18	14.5–15.8	1.68–1.73	0.8–1.0
8	12	15.2–15.9	1.70–1.76	0.8–1.3
10	6	15.6–15.8	1.73–1.77	0.6–1.0

* As in Table I.
Each filter-well contained 10 × 10⁶ cells.

Differential staining (Van Gieson) of sections similar to Fig. 4 H shows the aggregates to consist of epithelial cells surrounding central areas of connective tissue.

This centripetal migration of the cells has been found to occur with initial populations containing 20 million to 40 million cells per well, and the central nodule can be formed in as little as six days. Even with cell numbers within this range, migration to form a central nodule as shown in Fig. 4 A to D does not always occur, the cells forming instead discrete aggregates of varying size on the Millipore membrane (Dickson and Leslie, 1963b).

Reaggregation has also been found to occur with cells of other freshly dissociated organs in this system—notably with cells of adult human thyroid gland and human placenta (Dickson and Leslie, 1964). The cells of these organs become regrouped into numerous small nodules in which one cell type surrounds another; no annulus or central nodule is formed.

It is seen from these results that after disaggregation by enzymes the cells of an organ become reorganized and grouped according to type. Several workers have shown that such reorganization can occur with embryonic cells. For example, if an embryonic chick kidney is dissociated by trypsin and the cells are placed in medium in a gyrating flask they become sorted out according to their functional capacities and form small tubular structures and renal corpuscles (Moscona, 1961a; Steinberg, 1963). The capacity of the cells to reaggregate, however, has been found to decline with increasing age of the embryo, and as birth is approached and the cells become more differentiated the capacity is lost altogether (Moscona, 1961b; Garber, 1963). It was therefore believed that reaggregation would not occur in fully differentiated cells. The work with adult guinea-pig kidney cells just described was the first occasion on which reaggregation was shown to occur with adult cells. The filter-well therefore provided the appropriate conditions for these adult cells to reaggregate.

These experiments illustrate that the behaviour of a cell outside the body is influenced not only by the environmental conditions but also by the presence of other cell types. In fact it is only in such an organized cell system, in which all the cell types of the parent tissue are present, that we can expect the cells to behave and function as they do in the intact animal.

Epithelial-stromal Complex

The importance of this interdependence of cell types has been conclusively demonstrated in recent years by McLoughlin (1961a, 1961b). She used chick epithelium and mesenchyme and showed that the normal development and maintenance of epithelium was dependent on the underlying mesenchyme. By placing various kinds of mesenchyme in contact with the same type of epithelium she was able to alter the characteristics of the epithelium. For example, limb-bud epithelium in contact with limb-bud mesenchyme became cornified, but the same epithelium in contact with mesenchyme from the pancreas or salivary gland became secretory. McLoughlin concluded that on certain types of epithelium the mesenchyme exerted a continuous influence, without which the epithelial cells cannot survive, multiply, or differentiate, and postulated that the influence of the mesenchyme on the epithelium extended into adult life.

The possibility that epithelio-mesenchymal interactions continue into adult life has been confirmed by Lasfargues *et al.* (1960), who found that the mouse mammary carcinoma agent (Bittner milk agent) did not multiply in cultures of adult mouse mammary gland epithelium alone, but did grow in explants of mammary epithelium and stroma together. Franks and Barton (1960) observed that organ cultures of mouse prostate were under the control of testosterone, while

epithelial cells in the outgrowth from the cultures were not responsive to the hormone. They concluded that the epithelial cells were no longer under the influence of the fibromuscular stroma of the gland.

These few examples emphasize the importance of what I think is best termed the “epithelial-stromal complex” as a functional unit—that is, the association of epithelium and its adjacent connective tissue.

Cancer Cell in Relation to Stroma

It is a logical step in the discussion to consider the cancer cell in relation to its stroma, and several workers have emphasized the dependence of the cancer cell on a stromal element for survival and proliferation.

Leighton and Kline (1954) used small pieces of gelatin sponge to study cell interaction. It was found that cancer cells placed on the sponge did not survive, while fibroblasts grew into the interstices of the sponge. If a population of fibroblasts was allowed to grow into the sponge, and the cancer cells were then introduced, the malignant cells rapidly proliferated throughout the interstices.

Wolff (1962) devised a system in which he maintained cells in a fold of chick vitelline membrane on a semisolid medium. Human cancer cells died rapidly in this system unless they were placed in contact with a population of embryonic chick kidney cells on the opposite side of the membrane.

Other experiments probing epithelial-stromal relationships are those of Van Scott and Reinertson (1961) working with human basal-cell epitheliomas. They observed that isolates of tumour epithelium from basal-cell epitheliomas were unable to survive when transplanted autologously into the subcutis, though basal-cell epithelioma with stroma intact was able to survive and grow in the subcutis.

What is the situation with cancer cells in the filter-well? In experiments with cells from a malignant cell line (Hep 1) derived from a human cervical cancer it was found that they would not survive on the filter membrane beyond four days. When they were mixed with a population of cells from a non-malignant human liver line (HLM), however, they did survive.

It appears from our work and the work of others just described that the invasiveness and metastasis, and indeed the survival of the cancer cell, are governed by the connective-tissue stroma through which it is proliferating. This means that any method used for assessing the response of cancer cells to hormones or other chemotherapeutic agents must consider the cancer cell, not by itself, but in conjunction with the cells which it is invading. The implications of this are rather interesting, since it raises the question of whether there is a change in the connective-tissue cell as well as in the cell which becomes malignant. Such a change, leading to an imbalance in the relationship between epithelium and stroma, might even be involved in the initiation of malignancy. It has been suggested that the relationship between epithelium and its adjacent connective tissue is such a specific one in some tissues—for example, skin—that epithelial tumours do not metastasize beyond their place of origin to foreign tissue beds unless they are accompanied by their stroma (Pinkus, 1953). From the clinical point of view an alteration in the stroma could account for the different course of histologically similar cancer in two patients, one of whom dies within a few months, the other surviving for a year or more.

Hormone Responsiveness of Cells

From this discussion the need for a system which favours cell interaction is obvious if we are to study the behaviour and hormone-dependence of the cancer cell. It is also neces-

sary to demonstrate that cells will function in the system to the extent that cells of a target organ will respond to their stimulatory hormone.

Some months were spent investigating the hormone-responsiveness of cells of dissociated target organs in the filter-well, and sheep thyroid gland was eventually selected as the most suitable to study.

The cells of the thyroid gland are able to concentrate inorganic iodide from their surroundings (Fig. 5). The iodide is then oxidized to iodine and combined with the amino-acid tyrosine in stages to form iodinated amino-acids. These are: mono-iodo-tyrosine (3-iodotyrosine; MIT) containing one atom of iodine per molecule, 3,5-di-iodo-tyrosine (DIT) containing two atoms of iodine per molecule, and ultimately the hormone thyroxine (T4) with four atoms of iodine per molecule.

FIG. 5.—Formation of iodinated amino-acids in thyroid gland cells.

The accumulation of iodide by the thyroid can be conveniently demonstrated in the presence of a drug such as Tapazole (methimazole), which blocks the enzymatic oxidation of iodide and thus its combination with tyrosine but does not interfere with iodide transport into the gland cells. Under these conditions the normal thyroid maintains a concentration of free iodide 20 or more times that in the plasma, the actual value depending on the metabolic state of the thyroid cells (Berson and Yalow, 1955).

Iodide-concentrating Capacity of Cells

Table III shows the results obtained when sheep thyroid cells were maintained in filter-wells over a six-day culture period on medium containing radioactive iodide and the blocking agent Tapazole. Each filter-well in these experiments contained 40 million thyroid cells. The iodide-concentrating capacity of the cells is expressed as the C/M ratio, which is the number of radioactive counts (amount of radioactive iodide) per ml. of cells over the number of radioactive counts (amount

TABLE III.—Concentration of Radioactive Iodide by Sheep Thyroid Cells in Filter-Wells

Time in Days	No. of Cultures	Counts/ml. cells
		Counts/ml. medium = C/M ratio
2	8	6.4 ± 0.4
4	8	6.0 ± 0.3
6	8	7.0 ± 0.4

Radioactive iodide at an initial activity of 2 μCi/ml. Tapazole concentration 0.002 M. Each filter-well contained 40 × 10⁶ cells. C/M values are given as mean ± standard error.

of radioactive iodide) per ml. of medium. Initially the cells contained no radioactive iodide. It can be seen that by two days in culture the cells have reached a saturation plateau for iodide, the C/M ratio remaining constant from two to six days. The ratio in the region of 6–7 indicates that the cells are concentrating iodide to six to seven times the concentration in the medium under these conditions.¹

¹ Packed-cell volumes were read to the nearest 0.01 ml. in standard haematocrit tubes. The C/M values presented are not corrected for the volume of medium trapped in the packed-cell pellets. Extracellular space determined with ¹⁴C-inulin and ¹³¹I-albumin has been reported to amount to 49–60% of the packed-cell volumes (Tong, 1964); hence the true intracellular ¹³¹I-iodide concentrations were probably about twice those indicated by the uncorrected C/M values.

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Fig. 4.—Centripetal migration and reaggregation of adult guinea-pig kidney cells (37×10^6) in filter-well culture. Filter-wells (A to D), magnification 2. Sections (E to H) stained with haematoxylin and eosin.

A and E: Uniformly distributed plaque of cells on Millipore filter (A) and random arrangement of the cells at 4 hours in section (E). ($\times 100$.) B and F: Initial migration of the cells at four days to form a well-defined annulus (B) which on section shows elements of organization (F). ($\times 400$.) C and G: Continued centripetal movement of the cells at six days (C). The histological pattern shows further organization (G). ($\times 150$.) D and H: Formation of central nodule at 10 days (D). On section the nodule shows a highly organized structure in which epithelial cells enclose well-defined areas of connective tissue (H). ($\times 150$.)

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It has been shown clinically (Stanbury and Wyngaarden, 1952) and in experimental animals (Wyngaarden *et al.*, 1952) that certain monovalent anions, notably perchlorate and thiocyanate, can prevent iodide being concentrated in the thyroid gland and will also effect a discharge of any inorganic (unbound) iodide present within the thyroid cells. Perchlorate is the most potent of these compounds, and Table IV shows the result of adding a dilute concentration of sodium perchlorate to the medium of thyroid cultures 10 minutes before the end of the experimental period. It can be seen that the cells treated with perchlorate have discharged their iodide and the C/M ratio has fallen to the region of 1. A ratio in the region of unity is what would be expected if the cells were not concentrating the iodide but it was simply diffusing into them from the medium. In fact ratios in this region (1) are obtained when the iodide-concentrating capacity of cells other than thyroid—for example, cells from a liver line (HLM)—is measured.

TABLE IV.—Action of Sodium Perchlorate (NaClO₄) on Radioactive Iodide Concentration by Sheep Thyroid Cells in Filter-Wells

Time in Days	Culture	Counts/ml. cells Counts/ml. medium = C/M ratio	
		Counts/ml. cells	Counts/ml. medium
2	Control (4)	7.4 ± 0.27	
	NaClO ₄ (4)	1.6 ± 0.14	
4	Control (4)	6.7 ± 0.30	
	NaClO ₄ (4)	1.4 ± 0.12	
6	Control (4)	6.9 ± 0.22	
	NaClO ₄ (4)	1.3 ± 0.10	

Radioactive iodide at an initial activity of 2 µC/ml. Tapazole concentration 0.002 M. NaClO₄ concentration 0.002 M. Each filter-well contained 50 × 10⁶ cells. Figures in parentheses indicate the number of cultures studied.

Action of Thyroid-stimulating Hormone

In the intact animal the thyroid gland is under the control of the anterior pituitary by means of the thyroid-stimulating hormone (T.S.H.). The action of this hormone on sheep thyroid cells in filter-wells was next investigated, again with radioactive iodide as a marker. Table V shows the results of experiments in which the cells were again blocked by Tapazole and the C/M ratios compared in control and T.S.H.-treated cultures over six days. Each filter-well contained 40 × 10⁶ thyroid cells, and the concentration of T.S.H. was 0.14 unit/ml. It can be seen that T.S.H. had no significant effect on ¹³¹I uptake by the cells, the C/M ratios in the control and T.S.H.-treated cultures running parallel over the six days.

TABLE V.—Action of Thyrotrophic Hormone (T.S.H.) on Radioactive Iodide Concentration by Sheep Thyroid Cells in Filter-Wells

Time in Days	Culture	Counts/ml. cells Counts/ml. medium = C/M ratio	
		Counts/ml. cells	Counts/ml. medium
2	Control (8)	6.0 ± 0.2	
	T.S.H. (8)	7.0 ± 0.4	
4	Control (8)	6.5 ± 0.4	
	T.S.H. (8)	6.0 ± 0.3	
6	Control (8)	6.3 ± 0.2	
	T.S.H. (8)	5.9 ± 0.2	

Radioactive iodide at an initial activity of 2 µC/ml. Tapazole concentration 0.002 M. T.S.H. concentration 0.14 U/ml. Each filter-well contained 40 × 10⁶ cells.

The presence of Tapazole in the medium, as in the experiments just described, is very convenient from the experimental point of view for demonstrating iodide uptake by the thyroid cells, but it does not represent a very physiological state of affairs. Tong (1964) has postulated that T.S.H. may act by increasing the organification of iodide to the iodinated tyrosines, mono-iodo-tyrosine, di-iodo-tyrosine, and thyroxine, without necessarily affecting the iodide uptake by the thyroid cells. That is, Tong maintains that T.S.H. is increasing the formation of iodinated amino-acids from iodine and tyrosine and is acting beyond the stage at which Tapazole acts. If this is so, we would not have detected a T.S.H. effect in the experiments so far discussed.

The possibility that T.S.H. acts beyond the stage at which Tapazole produces its effect was investigated as follows.

Control cultures of thyroid cells and cultures with T.S.H. in the medium were set up as before, but this time in the absence of Tapazole (Table VI). The iodide taken up by the cells could therefore become incorporated into mono-iodo-tyrosine, di-iodo-tyrosine, and thyroxine. At the end of the culture period the radioactive iodide uptake by the cells was measured and expressed as a C/M ratio, the counts per ml. of cells this time representing organically bound as well as free iodide. It can be seen from Table VI that T.S.H. had no significant effect over the first two days. By four days, however, the hormone-treated cells contained 10 times the radioactivity present in the controls, and this increased to a twelvefold difference by six days.

TABLE VI.—Action of Thyrotrophic Hormone (T.S.H.) on Radioactive Iodide Concentration by Unblocked Sheep Thyroid Cells in Filter-Wells

Time in Days	Culture	Counts/ml. cells Counts/ml. medium = C/M ratio	
		Counts/ml. cells	Counts/ml. medium
2	Control (10)	8.7 ± 0.6	
	T.S.H. (10)	7.6 ± 0.3	
4	Control (10)	10.8 ± 0.8	
	T.S.H. (10)	100 ± 12.6	
6	Control (13)	15.4 ± 1.4	
	T.S.H. (13)	186 ± 26.5	

Radioactive iodide at an initial activity of 5 µC/ml. T.S.H. concentration 0.14 U/ml. Each filter-well contained 40 × 10⁶ cells.

These figures simply indicate an increased uptake of radioactive iodide into unblocked thyroid cells in the presence of T.S.H. They provide no information on the form in which the iodide is present in the cells.

Formation of ¹³¹I = Amino-acids

The fate of the iodide taken up by these unblocked thyroid cells was studied by digesting the cells with pronase (California Corp. for Biochemical Research). This enzyme hydrolyses the thyroid protein into its component iodinated amino-acids mono-iodo-tyrosine, di-iodo-tyrosine, and thyroxine, and these were then identified by paper chromatography and autoradiography, since they contain radioactive iodine.

The exact procedure was that at the end of the culture period the cells were digested with 0.5% pronase for six hours at 37° C. Known volumes of the resulting digest were then

FIG. 6.—Autoradiographs of chromatograms prepared from digests of thyroid cells cultured in the presence of ¹³¹I. Symbols on the left identify the components separated on the strips. C=Control population of cells. T.S.H.=Corresponding population of cells cultured in presence of thyrotrophic hormone (0.14 U/ml.).

spotted on paper chromatograms. The amino-acids were separated by running the chromatograms for 24 to 36 hours in a solvent system of collidine/3N ammonia (Tong *et al.*, 1962). Mono-iodo-tyrosine, di-iodo-tyrosine, and thyroxine were identified by spraying the chromatogram with ninhydrin and with diazotized sulphanilic acid (Taurog and Chaikoff, 1957). Autoradiographs were prepared from the chromatograms by placing an unexposed x-ray film in contact with the chromatogram in a cassette for four weeks. The radioactive iodine in the mono-iodo-tyrosine, di-iodo-tyrosine, and thyroxine caused localized exposure of the film and resulted in a series of dark bands on the negative.

In Fig. 6 are presented autoradiographs prepared as described from control populations of thyroid cells and populations cultured with T.S.H. in the medium.

The autoradiographs are in pairs, the left one of each pair being from the control population of cells, that on the right being from cells cultured in the presence of T.S.H. The exposure of the film is proportional to the amount of radioactivity present in the iodinated amino-acids on the chromatogram, and it can be seen that at each time interval the presence of T.S.H. has caused an increased incorporation of iodide into mono- and di-iodo-tyrosine, as shown by the darker bands produced by the T.S.H.-treated cells. It is also apparent that as the incorporation of iodide into mono- and di-iodo-tyrosine increases the inorganic iodide band becomes fainter.

It will be observed that there is a "tail" present on the autoradiographs produced by the four- and six-day T.S.H.-treated populations. This tail, we believe, is due to thyroxine, since it lies intermediate in position between the mono-iodo-tyrosine band and the inorganic iodide band and on the chromatogram it stains with diazotized sulphanilic acid and fluoresces under ultra-violet light. The thyroxine appears to have been incompletely separated from the inorganic iodide, possibly due to the particular conditions of chromatography employed.

Be that as it may, when the medium on which the thyroid cells were cultured is run on chromatograms and autoradiographs are prepared as with the cells, a definite band due to thyroxine appears in the autoradiograph produced by the medium from the T.S.H.-treated cells. In Fig. 7 are shown autoradiographs prepared from media on which a control

population and the corresponding T.S.H.-treated cells were maintained. The band due to thyroxine is clearly seen and is produced only by medium from the hormone-treated cells at four days and at six days.

From this series of experiments, therefore, it is seen that after enzymatic disaggregation of sheep thyroid gland the cells function to the extent that they will concentrate iodide, that the accumulated iodide can be discharged by perchlorate, and that the cells are capable of forming iodinated amino-acids mono- and di-iodo-tyrosine. The formation of mono- and di-iodo-tyrosine is greatly increased in the presence of T.S.H., which also stimulates the production of thyroxine by the cells.

It is worthy of note that no response to T.S.H. was obtained in populations of thyroid cells in the wells until hydrocortisone was added to the medium in dilute concentration (0.1 $\mu\text{g./ml.}$). It has been reported that hydrocortisone in dilute concentration helps to prevent cell degeneration *in vitro* by inhibiting protease release from the lysosomes (Fell and Thomas, 1961; Weissmann and Dingle, 1961).

Conclusion

To recapitulate, then, in the filter-well a population of cells obtained by enzymatic disaggregation of an organ will survive for 10 days or longer. The cells become regrouped according to type and produce a consistent and reproducible pattern of metabolism over the culture period. The cells of a target organ such as thyroid gland will carry out their characteristic function and will respond to their stimulatory hormone in the system.

Summary

In this lecture I have pointed out some of the difficulties facing us in the development of a laboratory method for assessing the response of cancer cells to hormones and other chemotherapeutic agents. The importance of cell interdependence has been emphasized, and in view of the previous failure by research workers to recognize the significance of the epithelial-stromal complex as a unit the inconsistency of results obtained to date in many so-called cancer chemotherapy screening tests is not surprising.

The filter-well technique, we believe, does show promise in this respect, since, as I have illustrated, it favours cell interaction and function.

FIG. 7.—Autoradiographs of chromatograms prepared from unhydrolysed media on which thyroid populations were cultured for four days and six days. Symbols on the left identify the components separated on the strips. C = Control medium. T.S.H. = Medium containing thyrotrophic hormone (0.14 U/ml.).

FIG. 8.—Glass unit for filter-well cultures. RV, open-necked reaction vessel with ground-glass flange; GL, glass lid with ground-glass flange; GT, glass taps; GJ, ground-glass joint with rubber cap; FW, filter-well; M, medium.

The wheel has now gone full circle as it were, and we are again in a position, but a much more confident position now, to investigate the hormone-dependence or otherwise of cancer in a target-organ.

The metal culture chamber of Trowell has been redesigned in glass, as shown in Fig. 8, the filter-well standing in medium directly in the bottom of the unit. The glass taps make the introduction and collection of gas from the unit more efficient, and elimination of the silicone-rubber sealing-ring has led to an increase in the amount of CO₂ recovered at the end of the culture period (Dickson and Leslie, 1966).

It is hoped eventually to establish a routine laboratory technique in which biopsy specimens from tumours will be disaggregated and their response to hormones and chemotherapeutic agents studied in such a filter-well system.

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Application of Plastic Surgery to the Care of the Injured*

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"Care of the Injured" is the basic theme of the lectures which are delivered annually at the Institute of Accident Surgery at Birmingham in memory of Alan Ruscoe Clark, who was devoted to this cause. "The injured" conjures up a diverse and diffuse class of patients abounding in complexities, complicated by infinitely variable factors resulting from different causes of injury. "The care" required is not confined to surgery or medicine, operating or resuscitation. It should commence with study to prevent injury at work, in the home, and on the roads.

It appears unusual for a plastic or dental surgeon to be consulted at an early stage in the treatment of injuries other than burns except at some specialized centres. More often plastic surgeons are called in to camouflage or tidy up disfigurements and disabilities which, in part at least, might have been avoided by earlier consultation. It should not require any

stretch of the imagination to associate plastic surgery with the care of the injured. It is the original and basic purpose of our specialty from the earliest writings of Susrata, about 700 B.C., through the Middle Ages to the time of Harold Gillies, Kelsey Fry, and Ivan Magill at the Cambridge Military Hospital, Aldershot, in 1915. This team was called upon to repair grievous missile wounds to face and jaws during the first world war. The second world war called for the repair of crashes, crushes, and burns, and it led to the regimentation of professional assets which resulted in an enlargement of interest and practice beyond the dreams of the original plastic surgeons.

This interest led to the correction of other deformities, congenital and acquired, the objective being restoration of normal function and appearance. Aesthetic considerations developed in surgery. This aspect of our work, important as it undoubtedly is, appeals to publicity and has been much over-emphasized. Nevertheless plastic surgery is now established in the United Kingdom and Commonwealth on a wide scale

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