BRITISH MEDICAL JOURNAL

LONDON SATURDAY FEBRUARY 10 1940

INTERACTION IN VITRO OF FIBROBLASTS AND SARCOMA CELLS WITH LEUCOCYTES AND MACROPHAGES

BY

R. J. LUDFORD, D.Sc., Ph.D.

Fellow of University College, London; Member of the Scientific Staff of the Imperial Cancer Research Fund

The study of tissue cultures of tumours has strikingly demonstrated what is also obvious in histological sections—that malignant tissues contain relatively large numbers of lymphocytes, monocytes, and macrophages. As to the role of these cells in tumour growth there has been a variety of opinions. Thus, Da Fano's (1912) histological study of spontaneously regressing tumours, and tumour grafts in resistant animals, led him to write of lymphocytes "that their intimate connexion with the development of immunity [to tumour growth] appears certain." Murphy's (1926) experimental studies led him to a similar conclusion—"that we have in the lymphoid elements an important link in the chain of the processes which determine resistance to cancer." Carrel investigated the problem by means of tissue culture. His work led him to a different conclusion—that "leucocytes secrete substances *in vitro* which promote cell multiplication." These substances he called "trephones." His evidence was derived from two types of experiments—growing leucocytes, together with fibroblasts, and also preparing extracts from leucocyte cultures and testing them on cultures of fibroblasts. When a fragment of spleen was grown close to a culture of fibroblasts it was observed that the fibroblasts, which after some days became enlarged and loaded with fat droplets, underwent a change when the leucocytes from the spleen culture reached them. They became slender and elongated, their cytoplasm increased in volume, and they began to proliferate actively (Carrel and Ebeling, 1923). Extracts of leucocytes were prepared by different methods. The blood of chicks was centrifuged and the leucocytes collected, washed, and stored in Ringer's solution in an ice-box for several days; then the suspension was shaken up, centrifuged, and the supernatant fluid applied to cultures of fibroblasts. Control cultures were treated with embryo extract, and others with Ringer's solution. The leucocyte extract was found to have the same growth-promoting property as embryo extract. Another extract was made from forty-eight-hours-old cultures of buffy coat grown on a clot of fowl plasma. Serum was extracted from this clot and also from another plasma clot incubated without leucocytes. The action of the two lots of sera was tested on cultures of fibroblasts. "The fluid from the medium which had contained leucocytes was markedly more active" (Carrel, 1922). The action of macrophages on the growth of malignant cells was demonstrated by explanting the two halves of a colony of rat sarcoma cells (Crocker rat sarcoma No. 10) some distance apart, and planting by the side of one a small piece of bone marrow. The sarcoma fragment in contact with the bone marrow

became partially surrounded by macrophages and grew vigorously, while the isolated fragment of sarcoma only grew feebly for a short time. Carrel also inoculated pure cultures of the sarcoma cells into the subcutaneous tissues of a rat. One culture was removed and explanted after twenty-four hours, before the sarcoma cells had become infiltrated with macrophages. Another culture was removed and explanted after four days. Macrophages had invaded this culture, and it grew more vigorously than the other (Carrel, 1927). In whatever manner lymphocytes may participate in the cellular mechanism involved in resistance to malignant growth the substantial evidence adduced by Carrel admits of only one interpretation—that leucocytes do not normally inhibit the growth of normal and malignant cells, but, on the contrary, stimulate their growth.

One of the most distinctive features of malignant tissues grown *in vitro* is the rapidity with which they digest clotted plasma. The possibility that increased liquefaction might also result from the stimulatory action of mononuclears led to this reinvestigation of the interaction of leucocytes with normal and malignant cells.

General Technical Procedure

All the tissue cultures employed in these experiments were grown in Carrel flasks. The culture media consisted of fowl plasma and chick embryo extract. The embryo extract was prepared by crushing to a pulp twelve chick embryos from eggs of nine to eleven days' incubation. This pulp was well stirred with 50 c.cm. of Tyrode's solution, then pipetted into centrifuge tubes. After allowing the tissue debris to settle, the volume of Tyrode's solution was made up to four times that of the tissue and stirred again. The tubes were centrifuged, and the supernatant fluid constituted the stock solution. This was diluted further with Tyrode's solution to the extent of 6 and 20 per cent.

In setting up cultures in large flasks (5 cm. in diameter) 0.6 c.cm. of plasma was pipetted into each flask; then 1.8 c.cm. of embryo extract was added and mixed with the plasma. Before clotting was completed the tissue fragments (approximately 1 sq. mm. in size) were introduced. For comparing the growth of fibroblasts and sarcoma cells (F) in the presence and in the absence of leucocytes (L) the tissue fragments were spaced as follows:

	F		F		F		L		L
F		F		L		L		L	
	F			L			L		L

Leucocyte cultures were prepared from the buffy coat of fowl blood by Carrel's method.

Growth of Fibroblasts in Presence and Absence of Leucocytes

When fibroblasts are explanted alone and also together with leucocytes it is found that in the latter case the growth of the fibroblasts begins sooner, is more extensive, and continues for a longer period. Also, the presence of leucocytes stimulates the fibroblasts to digest the plasma. This first experiment illustrates these phenomena. The tissues explanted were buffy coat from an adult hen and pectoral muscle from a two-weeks-old chick. This muscle tissue was used as a source of fibroblasts in preference to embryonic muscle, since cultures of the latter tend to be contaminated with myoblasts.

Cultures were set up as follows:

- A. 7 flasks, each containing 6 explants of buffy coat —42 explants
- B. 5 flasks, each containing 6 explants of pectoral muscle—30 explants
- C. 7 flasks, each containing { 3 explants of buffy coat —21 explants
3 explants of pectoral muscle—21 explants

On the following day embryo extract was added as nutrient medium.

By the fifth day it was obvious on superficial examination that the growth of fibroblasts in C was more extensive than in B. Many of the muscle explants in C had begun to liquefy the plasma. All the cultures were washed and re-fed.

On the ninth day all the fibroblast cultures of C were liquefied to varying extents. Liquefaction of fibroblast cultures had also begun in B. Some slight liquefaction of the buffy coat cultures of A had also occurred, the result of the disintegration of the explants. There was a distinct difference between the fibroblasts growing in the presence and those growing in the absence of leucocytes. The latter contained numerous fat droplets. Around the periphery of the cultures the cells appeared larger than those in the flasks containing both leucocytes and fibroblasts. In the latter cultures the fibroblasts had few or no fat droplets. The whole cultures appeared more translucent. The peripheral cells were spindle-shaped and radiated outwards into the surrounding medium. Areas of liquefaction were bordered by a zone of clear spindle-shaped fibroblasts. Liquefaction of the plasma of the flasks containing only fibroblasts did not begin until the cultures had almost attained their maximum growth, but in the flasks containing both fibroblasts and leucocytes liquefaction started earlier, and both liquefaction and growth were progressive.

On the twelfth day representative flasks from each group were fixed and stained. Three of these flasks are shown in Fig. 1. The first flask contains only cultures of leucocytes,

FIG. 1.—Twelve-days-old cultures of leucocytes (1), fibroblasts (2), and leucocytes together with fibroblasts (3), showing the more extensive growth and liquefaction of plasma when the two types of cells are grown together in contact.

the six explants being visible as large dots; the second flask contains six cultures of fibroblasts with small areas of liquefaction; while in the third flask are seen the large liquefied cultures of fibroblasts resulting from the presence of leucocytes.

By the eighteenth day most of the remaining cultures of leucocytes were degenerate, with cells filled with fat droplets. A few cultures still exhibited relatively healthy-looking monocytes, and there were a number of multinucleate giant cells present. All the cells in the flasks containing fibroblasts alone appeared degenerate. Very different was the state of the cells in the flasks containing both leucocytes and fibroblasts. Liquefaction of the plasma by the fibroblasts had continued; but the fibroblasts around the peripheries of the liquefied areas and others adherent to the bottom of the flasks in these areas were cells with clear cytoplasm with scarcely any fat droplets. They mostly retained their spindle shapes, except when flattened out on the surface of the glass. Owing to the extensive liquefaction of the plasma the buffy coat explants were displaced; but monocytes and macrophages, together with multinucleate giant cells, were scattered among the fibroblasts, and were also adherent to the surface of the flasks in the liquefied areas. These cells were obviously in a much more healthy state than those in the flasks containing only cultures of leucocytes. The extent of the liquefaction and growth of fibroblasts alone, and of fibroblasts with leucocytes, is shown in Fig. 2, which represents a reduction of

FIG. 2.—Tracings of eighteen-days-old cultures grown in flasks. I-III show the extent of growth and liquefaction of cultures of fibroblasts. IV-VIII show the greater growth and more extensive liquefaction in mixed cultures. In VIII are growths of fibroblasts (F) and leucocytes (L) on the surface of the glass in the liquefied area.

tracings made from each of the remaining flasks. The following table summarizes the progressive liquefaction of the cultures:

		No. of Cultures Liquefied			
		5th Day	7th Day	8th Day	9th Day
A. Leucocyte cultures	42 explants (7 flasks)	0	0	0	5 explants
B. Fibroblast cultures	30 explants (5 flasks)	0	0	0	3+ 14 starting
C. { Leucocytes ..	21 explants	0	—	—	—
+ Fibroblasts ..	21 explants (7 flasks)	9	16+ 2 partially	18	18+ 3 partially

Below is summarized another experiment in which the progressive growth of the cultures was measured with the aid of a graduated eyepiece. The units are therefore purely arbitrary, and the numbers given are significant only for comparative purposes. The average width of the outgrowth from each explant was recorded. From these readings was calculated the average width of the outgrowth from the fibroblast cultures, with and without leucocytes. In the table which follows these averages are given together with the number

of cultures liquefied at various intervals from the start of the experiment. The technique of the experiment was as follows:

First Day.—Similar tissues explanted in the same manner as in the previous experiment. *Second Day.*—Embryo extract was added. *Fourth and Eighth Days.*—Cultures were washed and re-fed.

Summary of Results

		6th Day	8th Day	9th Day	11th Day
Fibroblasts (alone): (42 explants)	Number of cultures liquefied	0	1 (slight)	1 (slight)	1 (slight)
	Average growth of cultures (x)	0.12	0.57	0.96	1.49
Fibroblasts (with leucocytes): (21 explants)	Number of cultures liquefied	1	10 begun	13 advanced	20 extensive
	Average growth of cultures (y)	1.14	2.09	2.62	Measurement not possible
Ratio y/x		9.5	3.7	2.7	

These figures demonstrate the more rapid initial growth of fibroblasts in the presence of leucocytes and also the early onset of liquefaction in such cultures. It must be pointed out, however, that it is not possible to measure with any degree of accuracy the extent of growth of cultures after liquefaction has begun.

Growth Stimulation and Liquefaction of Plasma

Reference has been made to Carrel's experiments with extracts from leucocyte cultures. This was repeated in a modified manner. Eight flask cultures of pectoral muscle and eight of buffy coat were prepared as previously described. Each flask contained eight explants. Two days after explantation embryo extract was added. Four days later the fluid was collected from the leucocyte cultures and allowed to stand for any tissue debris to settle down. Both leucocyte and fibroblast cultures were then washed with Tyrode's solution, and to four of the flasks containing fibroblast cultures 1.2 c.cm. of the fluid from the leucocyte cultures was added, and to the other four 1.2 c.cm. of embryo extract. There were thus thirty-two fibroblast cultures which had been re-fed with embryo extract and the same number re-fed with the fluid from the leucocyte cultures. There was no appreciable difference in the subsequent rate of growth of the sixty-four cultures or in the maximum size attained by them. Although the fluid from the leucocyte cultures had the same growth-stimulating properties as the embryo extract it did not induce liquefaction.

The possibility suggested itself that it might be some product of the metabolism of fibroblasts that was required by leucocytes to induce them to produce some substance with the property of stimulating proteolysis by fibroblasts. The following experiment was therefore performed.

The fluids from three flasks containing fibroblast cultures and from three flasks containing leucocyte cultures were collected separately four days after the first addition of embryo extract. The cultures in the six flasks were washed with Tyrode's solution. Then the fluid from the leucocyte cultures was added to the flasks containing the fibroblasts, and the fluid from the fibroblast cultures added to the flasks containing leucocytes. Four days later the fluids from the two different kinds of cultures were collected again separately. After washing the cultures with Tyrode's solution the fluid which originally came from the flasks containing the fibroblasts was returned to these flasks, and the fluid which originally came from the leucocyte flasks was returned to them.

After this procedure there was still no liquefaction of the plasma by either fibroblasts or leucocytes.

From these experiments it seems justifiable to conclude that leucocytes produce some substance or substances which stimulate the growth of fibroblasts, but that there is no evidence that the growth stimulation is responsible for the increased digestion of plasma which occurs in the presence of leucocytes. There is, of course, the possibility that leucocytes do produce some substance which is responsible for stimulating proteolysis but that this substance is extremely labile, or, alternatively, that my attempts to demonstrate such a substance failed because it was not present in a sufficiently high concentration. Examination of flasks of mixed cultures, however, has led me to believe that actual contact between leucocytes and fibroblasts is necessary in order to bring about the increased digestion of plasma. The experiment to be next described supports this view.

Cultures were prepared from pectoral muscle and spleen of a three-weeks-old chick. Embryo extract was added to all the flasks the day following explantation. From spleen explants there grew out in addition to fibroblasts lymphocytes, monocytes, and later macrophages. It was obvious during the first week that the growth of fibroblasts was more rapid and more extensive from fragments of spleen. The various cultures in the flasks grew separately as isolated colonies of cells. The condition of the colonies on the twelfth day is summarized below:

Muscle explants alone	Growth of fibroblasts	40 cultures (6 flasks)	No liquefaction
Spleen explants + leucocytes	Growth of fibroblasts + leucocytes	18 cultures	14 completely and 4 partially liquefied
Muscle explants	Growth of fibroblasts	21 cultures (6 flasks)	No liquefaction

In other experiments in which spleen and muscle explants have been grown close together, and leucocytes from the spleen cultures have infiltrated the sheets of fibroblasts, the latter have digested the plasma. These experiments therefore confirm the conclusion that, while leucocytes produce substances that stimulate the growth of fibroblasts, actual contact between the two types of cells is necessary to cause an increase of the fibroblasts' proteolytic capacity.

Interaction of Leucocytes and Sarcoma Cells

For investigating the action of leucocyte cultures upon sarcoma cells I have employed Gye's fowl sarcoma R.F.D.2, which was originally induced in a fowl by the injection of dibenzanthracene. When this tumour is explanted there is a considerable outgrowth of monocytes and macrophages. After subculturing several times these cells are eliminated, leaving pure cultures of the sarcoma cells. Considerable difficulty has been experienced in maintaining such cultures for any length of time. It has been found that after the elimination of the monocytes and macrophages the sarcoma cells do not liquefy plasma much more rapidly than do normal fibroblasts.

Growing buffy coat cultures together with cultures of the sarcoma does not accelerate appreciably the rate of or increase the extent of proteolysis by this tumour. In my experiments there would appear to have been enough monocytes and macrophages in the primary tumour explants to induce the maximum stimulation of proteolysis. When, however, leucocytes and sarcoma cultures were grown together in flasks there was more extensive growth of the malignant cells than when sarcoma cultures were grown alone. In the following experiment that

increased growth of fibroblasts and sarcoma cells in the presence of leucocytes was compared.

The tissues explanted were pectoral muscle from a two-weeks-old chick, buffy coat, and sarcoma R.F.D.2. These tissues were explanted thus:

Three flasks each of:

- A. 6 explants of pectoral muscle
- B. 3 explants of pectoral muscle plus 3 explants of buffy coat
- C. 6 explants of sarcoma
- D. 3 explants of sarcoma plus 3 explants of buffy coat

Embryo extract was added to each flask on the second day. All the flasks were washed and re-fed on the eighth day. Three weeks from the beginning of the experiment, after all growth had ceased, the total width of each culture of muscle and of sarcoma was measured. From these figures were calculated the average diameters of the cultures of fibroblasts and sarcoma cells, alone and in the presence of leucocytes. These were:

A. Fibroblasts alone	9.2 mm.	} 1:1.4
B. Fibroblasts + leucocytes	13.0 mm.	
C. Sarcoma	5.4 mm.	} 1:1.3
D. Sarcoma + leucocytes	7.2 mm.	

The results of a repetition of this experiment, in which only sarcoma R.F.D.2 alone and together with buffy coat was grown, are given in the table below. The measurements were made in arbitrary units in the manner described in a previous experiment. In addition to measuring the outgrowth of sarcoma cells the outgrowth of the wandering cells was recorded.

		3rd Day	5th Day	9th Day	13th Day
Sarcoma alone: 42 explants (7 flasks)	No. of cultures liquefied	0	14%	50%	60%
	Growth of sarcoma cells	0.7	1.5	2.7	2.8 (1)
	Outgrowth of monocytes and macrophages	1.0	1.5	2.4	2.2*
Sarcoma: 27 explants in 9 flasks, with equal number of buffy coat explants	No. of cultures liquefied	0	5%	25%	56%
	Growth of sarcoma cells	0.6	1.4	3.2	3.9 (1.4)
	Outgrowth of monocytes and macrophages	0.8	1.5	1.8	1.8

* Less than previous figure owing to degeneration.

In this experiment the buffy coat cultures grew quite apart from the sarcoma cultures. The table shows that there was no increase of liquefaction by the sarcoma cultures. Liquefaction was actually slower. This was possibly the result of the slower rate of outgrowth of the wandering cells. A possible explanation of the greater size ultimately attained by sarcoma cultures in the presence of buffy coat is that the macrophages from the latter remain in an active functional state for a longer period.

In old cultures of leucocytes and sarcoma cells the latter appear more healthy than when grown alone. This is more obvious when the culture medium is not changed for a long time. The more active physiological state of the cells under these conditions is illustrated by the following experiment, in which old cultures were inoculated into chicks.

Small Carrel flasks (1.25 cm. in diameter) were employed. Into each was introduced 0.9 c.cm. of 6 per cent. embryo extract and 0.3 c.cm. of plasma. There were explanted six flasks each of:

- A. 4 explants of pectoral muscle of a two-weeks-old chick
- B. 2 explants of muscle plus two explants of buffy coat
- C. 4 explants of R.F.D.2 sarcoma
- D. 2 explants of sarcoma plus two explants of buffy coat

Two days later 0.4 c.cm. of 20 per cent. embryo extract was added to each flask. They received no further treatment except daily tilting backwards and forwards to keep the fluid uniform. On the sixty-first day the cultures were measured in arbitrary units as previously described. The averages then were:

A. Fibroblasts (alone)	6.2
B. Fibroblasts with leucocytes	8.8
C. Sarcoma (alone)	4.3
D. Sarcoma with leucocytes	6.4

By this time the cultures had obviously passed their prime, but the cells in the mixed cultures were clearly in a better state than in the others. On the next day the three flasks with the best cultures of sarcoma cells were chosen from the flasks containing sarcoma alone and sarcoma with leucocytes. The plasma clots were removed from the flasks and the sarcoma cultures cut out. These cultures were inoculated into the breast muscle of young chicks: three cultures from each flask containing sarcoma alone into the left breasts; three cultures from the flasks containing mixed cultures into the right breasts. The three latter all gave rise to tumours which grew progressively. Only one tumour grew as the result of the three inoculations into the left breast, and this tumour grew much slower and was approximately one-quarter of the size of the others when it became necessary to kill the chicks. This affords clear evidence that the sarcoma cells were in a more active state in the mixed cultures. The surprising fact about this experiment is that sarcoma cells should have survived under such unfavourable physiological conditions in cultures which had received no treatment for two months.

Discussion

The experiments described confirm the work of Carrel in that they demonstrate that leucocytes produce substances which stimulate the growth of fibroblasts and sarcoma cells. When fibroblasts are grown with leucocytes, growth begins sooner and continues longer. These experiments have also shown that contact between leucocytes and fibroblasts stimulates the proteolytic properties of the latter. When these two types of cells are grown together in flasks, growth and digestion of the plasma continue until the greater part of the plasma is liquefied. This goes on in flasks which are untreated for long periods. The conditions in these flasks are such that the two types of cells flourish in a sort of symbiotic relationship, in that both appear to find more favourable conditions for their growth than when cultured separately. The growth of the sarcoma cells employed has not been found to attain the same proportions in mixed cultures as do cultures of fibroblasts grown under the same conditions. This difference is probably due to the greater accumulation of toxic metabolic products, such as lactic acid, in the sarcoma cultures.

I have referred throughout to the cells growing from explants of buffy coat as leucocytes. Since the granulocytes disappear at an early stage from these cultures there remain lymphocytes, monocytes, and macrophages, which are regarded by many as different developmental phases of a common cell type. These would seem to be the cells responsible for the activity exerted by the buffy coat cultures. Older cultures of buffy coat contain a few monocytes, numerous macrophages, and some multinucleate giant cells. The non-malignant cells which migrate from explants of sarcoma are mostly monocytes and macrophages. Further investigations are required to determine whether lymphocytes, monocytes, and macrophages all alike contribute to produce the effects which have been described.

On the basis of the results of this work it seems highly probable that the monocytes and macrophages of tumours

may have an appreciable influence on their growth *in vivo*. In this connexion it is pertinent to point out that when pure cultures of malignant cells are grown side by side with cultures of normal tissues, infiltrative growth is very slow or does not occur at all. Earle (1937), in comparing the slow infiltration of fragments of muscle by the Walker rat carcinoma No. 256 *in vitro* with "the extreme rapidity of infiltration and destruction of surrounding tissues by the tumour grown *in vivo*, particularly in intramuscular injections," remarks that "there are as yet important, unanalysed factors which influence or control the infiltration of other tissues by the living tumour cell." The results of my experiments suggest that these factors may be the presence of monocytes and macrophages in the tumour *in vivo* which are absent from pure cultures growing *in vitro*. One naturally hesitates before applying results obtained from experiments *in vitro* to the explanation of processes occurring in the animal body. So far as I am aware, however, there is no record in the literature of a specific cellular reaction occurring *in vitro* which has not also been found to occur under appropriate conditions *in vivo*. One might expect, therefore, that the results of interaction between leucocytes and macrophages with malignant cells in the animal body would be of the same kind as in tissue cultures. On this assumption one might expect that leucocytes and macrophages would stimulate not only the growth of the malignant cells but also their proteolytic capacity, and thereby facilitate the infiltration of normal tissues.

Summary

The results of these experiments confirm the work of Carrel in demonstrating that cultures of leucocytes produce substances which stimulate the growth of fibroblasts and sarcoma cells.

When fibroblasts or sarcoma cells are grown together with leucocyte cultures their growth begins sooner and is ultimately more extensive than when they are grown alone. Growth stimulation occurs without actual contact between the two types of cells.

When fibroblasts or sarcoma cells are grown in contact with the cells from leucocyte cultures not only is the growth of the former stimulated but also their capacity to digest clotted plasma (proteolysis).

In mixed cultures both kinds of cells remain in a more active functional state.

On the assumption that the same cellular reactions occur in the animal body as have been described in tissue cultures, it is suggested that monocytes and macrophages by stimulating the growth and proteolytic activity of malignant cells may facilitate their invasive growth into normal tissues.

REFERENCES

- Carrel, A. (1922). *J. exp. Med.*, **36**, 385.
 — (1927). *C. r. Soc. Biol.*, Paris, **97**, 19.
 — and Ebeling, A. H. (1923). *J. exp. Med.*, **38**, 513.
 Da Fano, C. (1912). 5th. Sci. Rep. of the Imp. Cancer Res. Fund, p. 57, London.
 Earle, W. R. (1937). *Arch. exp. Zellforsch.*, **20**, 140.
 Murphy, J. B. (1926). Monographs of the Rockefeller Inst. for Med. Res., No. 21, New York.

Prolonged Coma.—According to Lester prolonged coma occurring in the course of insulin shock therapy is a complication which should be avoided whenever possible, as it is not usually followed by improvement in the patient's mental state. Such improvement as may be obtained in a few cases does not justify attempts to produce prolonged coma, as the mortality rate is too high (16 per cent. in this series).—Study of Prolonged Coma following Insulin Shock. D. Lester.—*Amer. J. Psychiat.*, March, 1939, **95**, 1083.

EFFECT OF OESTRADIOL BENZOATE ON THE AMOUNT OF GONADOTROPHIN FOUND IN THE PITUITARY GLAND AND URINE OF POST-MENOPAUSAL WOMEN

BY

I. W. ROWLANDS, Ph.D.

National Institute for Medical Research, Hampstead

AND

E. P. SHARPEY-SCHAFER, M.R.C.P.

British Postgraduate Medical School, Hammersmith

The effect of oestrogens on the gonadotrophic activity of the pituitary gland has been studied by several methods. First, observations have been made on the gonads of the injected animal or on the gonads of another animal united to it in parabiosis (Martins, 1929; Kallas, 1930; Bunster and Meyer, 1938). These methods are not applicable to man. Secondly, the amount of gonadotrophic substance present in the urine and blood has been investigated. Frank and Salmon (1935) and Jones and MacGregor (1936) found that the gonadotrophic substance disappears from the urine of ovariectomized or post-menopausal women treated with oestrogen. Thirdly, indirect information has been obtained from the histology of the pituitary gland (Severinghaus, 1939). Fourthly, the amount of gonadotrophic hormone in the anterior pituitary gland has been estimated directly. Several workers (Meyer, Leonard, Hisaw, and Martin, 1930, 1932; Bialek-Laprida, 1933) found a diminished amount of gonadotrophic hormone in the pituitary glands of gonadectomized rats which had been treated with oestrogen. Henderson and Rowlands (1938) and Noble, Rowlands, Warwick, and Williams (1939) showed that there is an increased amount of gonadotrophic hormone in the pituitary gland of post-menopausal women. In this paper we describe the effect of the injection of large amounts of the oestrogenic substance oestradiol benzoate on the pituitary gland of similar subjects.

Methods

Administration of Oestrogen.—Four patients, whose ages ranged between 54 and 70 years, were injected intramuscularly daily with 10 mg. (100,000 I.B.U.) of oestradiol benzoate in oil solution. One patient was injected with 30 mg. daily for three days. These five patients were suffering from incurable disease, and the cause of death in the order shown in Table II was right heart failure, cirrhosis of the liver, chronic nephritis, carcinoma of the breast, and carcinoma of the cervix. At the post-mortem examination the endocrine glands appeared normal in each case. The pituitary gland was removed and the anterior lobe weighed, desiccated in acetone, and afterwards dried in a stream of hot air. Each gland was then ground to a fine powder and weighed. The pituitary glands of five other women of about the same age who had not been treated with oestrogen were used as controls.

Extraction of Urine.—Another woman, who had been ovariectomized twenty-five years previously, was injected with the same amount of oestradiol benzoate for six days. The urine of this woman was collected for three periods—namely, before, during, and ten days after the injections ceased. Crude extracts, which are referred to as CU1A,