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EXPERIMENTS ON CANCER-PRODUCING SUBSTANCES.

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(From the Cancer Hospital Research Institute, London.)

(With Special Plate.)

In a previous paper¹ the production of cancer by a mixture of compounds formed from isoprene

at about 820° C. was recorded. In the present paper further experiments on the formation by heat of cancer-producing materials are described. The nine products tested were obtained from the following sources at, approximately, the temperatures* stated: (1) acetylene (700°); (2) acetylene (800-900°); (3) Californian petroleum (880°); (4) isoprene (700°); (5, 6, and 7) Durham Holmside coal (450°, 560°, and 1,250°); (8) human skin (920°); (9) yeast (920°).

Technique.

These substances, with the exception of the coal, were exposed to heat in the manner described in the earlier paper.¹ A silica tube about 36 in. by 1 in. is filled with pieces of porous plate and heated in a gas furnace; the temperature is ascertained by means of a nichrome thermocouple inserted into an inner silica tube which reaches nearly to the middle of the larger one. When the temperature required has been reached, the gas, liquid, or vapour derived from solid materials is passed through the heated tube and the condensable products collected in water-cooled receivers. Further details are given under "Skin Tar" below. By this method the temperature at the point of the thermocouple is measured very accurately, but it is not possible to control the temperatures to which the vapours may be exposed in all other parts of the tube; hence the temperatures stated in this paper are to be taken as approximate only.

The tarry products were applied twice weekly to mice (generally 100 for each test) in the interscapular region.

ACETYLENE TAR.

A compound even simpler than isoprene—namely, acetylene $\text{HC}\equiv\text{CH}$ —can be used as a source of cancer-producing compounds. Berthelot² in 1866 showed that a tarry material containing benzene is formed when acetylene is heated; this synthesis of benzene is, of course, of great importance in the history of organic chemistry. He says:

"En chauffant l'acétylène pur, dans une cloche courbe, sur le mercure, à la température de ramollissement du verre vert, on voit ce gaz diminuer peu à peu de volume. En même temps des produits goudronneux apparaissent. . . . Dans une expérience, au bout d'une demi-heure, 97 centièmes de l'acétylène primitif avaient disparu, 3 centièmes seulement subsistant. . . . Après une suite fastidieuse de manipulations méthodiques j'ai obtenu en quantité suffisante un liquide jaunâtre que j'ai soumis à une distillation fractionnée. . . . j'ai isolé toute une série de carbures d'hydrogène, polymères de l'acétylène (benzène, styrolène, hydrure de naphthalène, hydrure d'anthracène, carbures fluorescents, etc.) . . . La benzène forme près la moitié du produit total."

Recently Zelinsky³ has shown that these condensation products of acetylene can be prepared in much greater quantities if the gas is passed through a heated tube filled with wood charcoal; about 70 to 74 per cent. of the acetylene is then converted into "goudron acétylénique." He obtained by this method as much as 180 grams of liquid in eleven hours at 640° to 650°; the compounds identified were benzene, toluene, paraxylene, styrol, indene, naphthalene, fluorene, and anthracene.

These "produits goudronneux" of acetylene, discovered by Berthelot sixty years ago, have considerable cancer-producing power (Figs. 1 and 2). In the series of experiments described here acetylene, generated in the ordinary way from calcium carbide, was purified by bubbling through watery solutions of (1) copper sulphate and sulphuric acid (two flasks), (2) mercuric chloride and hydrochloric acid, and (3) caustic soda, and then passed into the silica tube. The tube was charged in the earlier experiments with wood charcoal as in Zelinsky's method; the charcoal was first extracted with ether to remove any traces of wood tar which might possibly be present. Later it was found that porous plate could be used, and with the latter material stoppage of the tube by deposition of carbon from the gas occurred much less frequently. The tar is a mobile dark brown liquid smelling of benzene; the yield in these experiments was generally not more than 4 or 5 c.cm. an hour. Two different products made at 700° and at 800° to 900° respectively were used.

Acetylene Tar (700°).—The two first tumours appeared on the 86th day; the first section showing a malignant growth was obtained on the 153rd day (Fig. 1). By the 258th day 15 tumours had been obtained: of these, 13 were undoubted cancers in which the epithelium reached or penetrated the panniculus carnosus; the other 2 were papillomata with some downgrowth.

Acetylene Tar (800° to 900°).—The first tumour appeared on the 105th day. This tar was certainly less effectual than the 700° tar; unfortunately there was a high mortality in the 800° to 900° series which made comparison more difficult. On the 125th day the 700° series consisted of 28 mice, of which 13 bore tumours, and the 800° to 900° series of 14 mice, of which only 2 bore tumours. The experiment was not continued after the 116th day as it was clear that the 800° to 900° tar was inferior to the other; this series of mice, and another painted subsequently, with tar made at the same temperature, yielded in all 8 cancers and 6 papillomata with downgrowth (Fig. 2). Altogether, in these and some other series of mice to be reported later, 30 cancers have been produced with three different preparations of acetylene tar. This acetylene tar resembles isoprene tar in the comparative simplicity of its composition; elements other than carbon and hydrogen can be present in it in traces only. It therefore offers for the study of cancer production a material less complex than coal tar, for in the latter oxygen, nitrogen, and sulphur are present also. The samples of acetylene tar prepared hitherto seem to be less active than isoprene tar (820°) in causing the growth of tumours; but the former has the great advantage of being easier to prepare in quantity. A chemical study of acetylene tar is now in progress in this Institute.

* All temperatures referred to in this paper are on the centigrade scale.

HEATED PETROLEUM.

In this experiment a petroleum having no cancer-producing power was found to acquire this power when heated.

The Californian petroleum used had been tested in the unheated state by Dr. Leitch, and found by him to have no cancer-producing action; in his experiment 100 mice were used, and although the painting was continued until the 515th day, when the last mouse died, no tumours were obtained. A sample of this oil was then passed at the rate of 12 to 15 c.cm. an hour over porous plate in the silica tube at about 880° in a current of hydrogen; the product is darker and more viscous than the original oil, and smells of benzene. The first tumour appeared on the 67th day after nineteen applications of this material; the first sections showing malignancy were obtained on the 170th day (Fig. 3). The experiment was not continued after the 206th day, when 10 mice, all bearing tumours, remained alive. Altogether 15 tumours were examined microscopically; of these, 11 were certainly malignant, for the downgrowing epithelium reached or penetrated the panniculus carnosus, and the remaining 4 were papillomata.

The importance of heat in the formation of cancer-producing substances is shown very clearly by this experiment. In the petroleum industry the changes brought about in the oil by heating, including the conversion of aliphatic into aromatic compounds (anthracene, phenanthrene, pyrene, retene), are well known, and have been the subject of many patents. The experiment shows also that the products of petroleum most dangerous to workmen must be any strongly heated distillation residues; two out of the few fully recorded cases of petroleum cancer in the literature⁵ occurred in men exposed to such materials.

ISOPRENE TAR (700°).

The isoprene tar described in a previous paper¹ was made at 820°. Staudinger, Endle, and Herold, who first prepared such material,⁶ found considerable chemical differences between tars prepared from isoprene at 700°, and at 800° to 820°; the former consisted chiefly of unsaturated, the latter chiefly of saturated aromatic, compounds. One might expect, then, that a comparison of the cancer-producing power of two such tars would indicate the class of compound which causes the growth of tar tumours. Accordingly, an isoprene tar was made at 700° to 720°, the technique being in other respects the same as that of the earlier preparation at 820°. The 700° to 720° tar differed from the 820° tar in being more liquid, and in retaining a faint smell of isoprene; in causing new growth it was certainly inferior. The rates of production of tumours by the two tars are compared in Table I.

TABLE I.—Isoprene Tars (700° and 820°).

		Number of Mice Alive—			
		With Tumours.	Without Tumours.	Total.	Percentage with Tumours.
Day 87:					
Isoprene tar 700°	...	5	41	46	11
" " 820°	...	14	27	41	35
Day 132:					
Isoprene tar 700°	...	3	16	19	16
" " 820°	...	21	7	28	75
Day 168:					
Isoprene tar 700°	...	3	15	18	17
" " 820°	...	19	1	20	95

TARS MADE AT KNOWN TEMPERATURES (450°, 560°, 1,250°) FROM COAL.

Deelman⁷ tested tars from the Amsterdam gasworks, and found that the horizontal retort tar produced cancer more rapidly than the vertical retort tar; the latter variety of tar undergoes less exposure to the retort temperature, and tends to show the chemical characters of a low temperature tar (higher content of paraffins and phenols, lower content of non-phenolic aromatic compounds).⁸ Experiments with horizontal and vertical retort tars (for which material I am indebted to the Gas Light and Coke Company) did

not give quite conclusive results. But it is known that different coals may yield tars differing considerably in chemical composition (see, for instance, Börnstein⁹); in such experiments it is therefore necessary that the tars compared should be derived from the same sample of coal. Through the kindness of the Fuel Research Board and of the staff of H.M. Fuel Research Station at Greenwich, some material very well fitted to the object in view was obtained—namely, three tars made from Durham Holmside coal at 450°, 560°, and 1,250°; the last of these would correspond most nearly to the ordinary gasworks tar.

TABLE II.—Tars from Durham Holmside Coal. Numbers of Mice Alive with and without Tumours.

Day.	450°.		560°.		1,250°.	
	With Tumours.	Without Tumours.	With Tumours.	Without Tumours.	With Tumours.	Without Tumours.
138th	1	90	11	55	11	52
178th	4	69	14	30	18	20
230th	2	47	10	19	10	10
290th	5	22	11	6	6	5
373rd	5	6	4	—	2	1

The experiment was stopped on the 373rd day.

The results given in the table show that the 450° tar was much inferior to the two others in tumour production. Thus on the 230th day the tumour-bearing mice made up 50 per cent. of the 1,250° series, 33 per cent. of the 560° series, and only 4 per cent. of the 450° series. Seven tumours from the 450° mice were examined microscopically; there was little or no downgrowth in six of these, in the seventh the epithelium reached the panniculus carnosus, and must be regarded as malignant in character. From each of the two other series 6 epitheliomata were obtained, the remaining tumours from these two series being, in all, 15 papillomata, of which 13 showed downgrowth.

The comparison of such batches of mice is subject to many sources of error, but this experiment seems to demonstrate (1) that the 450° tar is much less active than the other two, and (2) that the 1,250° tar is somewhat more active than the 560° tar. The results, then, indicate that, when coal is carbonized, (1) the cancer-producing agent is being formed in very small amount even below 450°; (2) a rapid increase in its formation takes place between 450° and 560°; and (3) this increase continues, but at a much slower rate, from 560° to 1,250°; in other words, the curve expressing the formation of this agent with rising temperature ascends at first very steeply from the region about 450° and proceeds much less steeply from 560° to 1,250°.

TARS DERIVED FROM YEAST AND FROM HUMAN SKIN.

Until recently all the cancer-producing substances available for experiment have had their origin in more or less remote geological periods; they have been derived from the coal or oil-shales of the Carboniferous period, from the Oligocene lignite beds, or from petroleum-bearing strata ranging from the Devonian to the Miocene. But is it necessary to go so far back into the past for such materials? The laboratory product isoprene is actually derived, by way of turpentine oil or caraway oil, from plants now living. Experiments were carried out to ascertain whether other plants and animals living at the present day could yield compounds which produce cancer.

The two materials selected for the first experiments were: (1) yeast, representing the unicellular organisms in which no malignant growth can occur, and (2) human skin, including the epidermis, in which cancer may arise naturally.

Skin Tar.

Human skin was obtained from amputated breasts and limbs, and from the margin of the incision at autopsies; in subjects affected with cancer skin having any appearance of infiltration with the growth was rejected.

The skin was freed as far as possible from adipose tissue, cut into small pieces, and dried in a vacuum desiccator over calcium

FIG. 1.—Acetylene tar (700°): 177th day. Invasion of voluntary muscle. ($\times 150$.)

FIG. 2.—Acetylene tar (800°-900°): 216th day. Two nodules of epithelioma lying in voluntary muscle. ($\times 100$.)

FIG. 3.—Heated Californian petroleum (880°): 170th day. Invasion of voluntary muscle. ($\times 350$.)

FIG. 4.—Skin tar (920°). Mouse on 206th day, after 58 applications. Section showed epithelioma.

FIG. 5.—Skin tar (920°). Mouse on 216th day. Section showed downgrowth just reaching the panniculus carnosus.

FIG. 7.

FIG. 8.

FIGS. 7 AND 8.—Skin tar (920°); 172nd day. (Fig. 7, $\times 25$; Fig. 8, $\times 50$.)

FIG. 6.—Skin tar (920°). Mouse on 227th day.

FIG. 9.—Skin tar (920°); 208th day. Epithelioma penetrating panniculus carnosus. ($\times 100$.)

FIG. 10.—Yeast tar (920°); 182nd day. ($\times 150$.)

chloride. Microscopic sections of such skin before drying show that the epidermis may occupy as little as one-fiftieth of the area of the section; all the rest consists of the fibrous tissue of the dermis. Hence it may be that only a very small fraction of the tar obtained from such skin is derived from the epidermis. A current of hydrogen, purified by passing through solutions of potassium permanganate and sulphuric acid, silver nitrate, and caustic soda, was passed through a silica tube 36 in. in length, of which the proximal one-third was filled with dried skin (about 45 grams) and the distal two-thirds with porous plate; the temperature near the middle of this distal portion was read by means of the thermocouple. The temperature was first raised to the degree selected (920° or 780°), and heat was then applied very gently to the proximal portion of the tube containing the skin, which was thus decomposed slowly (45 grams of dried skin in six to seven hours); the volatile products passed over the hot porous plate and the condensate (solid ammonium carbonate and carbon, watery fluid, and tar) was collected in cooled receivers, and subsequently shaken with water and ether. The ethereal layer was shaken twice with water to remove ammonia and a part of the pyridine, the ether evaporated *in vacuo*, and the black oily residue was applied to a series of 50 mice twice weekly.

Skin Tar (920°).—The first papilloma appeared on the 53rd day, and the first specimen showing malignant growth microscopically was obtained on the 128th day. On the 198th day 13 out of the 16 mice alive bore tumours. In all, at the time of writing (233rd day) sections of 4 cancers and of 5 tumours in which the downgrowth reaches, or nearly reaches, the panniculus carnosus, have been obtained; while of the 8 mice alive 5 bear tumours which have the appearance of malignancy (Figs. 4 to 9).

[Note added at Correction of Proof.—At the present date (June 22nd, 1925) total numbers of tumours produced are:

		Cancers.	Papillomata.
Acetylene tar		36	15
Skin tar (920°)		12	8
Yeast tar (920°)		3	2]

These experiments with skin tar seem to be the first in which a cancer-producing material has been obtained by any treatment of human tissues. One cannot, of course, assume that these cancers of the skin produced by a tar derived from skin are in any way more closely allied in origin to the naturally occurring epitheliomata than are cancers produced by a tar derived from the flora of the Coal Age; at the present stage of the investigation one cannot even form any opinion upon this point. However, it is not wholly impossible that the living skin may be capable of forming very slowly substances which, when required in bulk and rapidly, can *in vitro* be produced from skin only at a red heat; many instances are known of the ease with which the body or its enzymes bring about chemical changes which otherwise demand strong reagents or high temperatures—for example, the hydrolysis and oxidation of fats and proteins. In making cancer-producing material from skin we expect to obtain a good supply of it in a few hours, and must hence use very vigorous methods; it is possible that the body at its own temperature takes months or years to produce a quantity of some substance sufficient to influence the growth of a few cells only.

Yeast Tar (920°).

Compressed yeast was treated in exactly the same manner as the human skin; the products in the receivers contained more water and less ammonia than did those from the skin, but the two tars showed no obvious difference. Most unfortunately there was a high death rate among the mice painted with 920° yeast tar; this makes difficult any exact comparison with the tar made from skin at the same temperature, but there is no doubt that the yeast tar was the slower in action. Thus, on the 182nd day, 11 out of the 18 mice in the skin series, and only 1 out of the 9 mice in the yeast series, showed tumours. The first tumour was situated underneath the epidermis; it was noticed on the 178th day, and the mouse was killed four days later. The tumour (Fig. 10) was an epithelioma. Sections from another mouse (229th day) showed a tumour with downgrowth approaching the panniculus carnosus. At the time of writing (238th day) 3 out of the original 50 mice are alive; all bear tumours, and of these one is almost certainly malignant.

DISCUSSION.

1. It may be said that these pyrogenous materials derived from acetylene, isoprene, petroleum, yeast, and skin produce cancer simply because they are all "irritants"; and that the multiplication in the laboratory of instances of "chronic irritation" is not required. To apply thus to a substance, after it has been shown to produce cancer, the term "irritant" gives an appearance of explanation which is illusory; we only know that the substance produces cancer. There seems to be a tendency at the present time to state that a substance such as soot gives rise to cancer because it is an irritant, and at the same time tacitly to regard it as an irritant simply because it gives rise to cancer; this circular treatment of the matter is of no value at all. Would anyone have thought of singling out soot as an irritant if its carcinogenic power had happened to remain unknown?

Again, the undoubted irritants which do not produce cancer are so numerous that the same term cannot usefully be applied to define the action of other agents, some of them not obviously irritating in character, which do produce cancer. Thus blast-furnace tar, and some petroleum which give negative results in mice, do far more visible damage to the skin than acetylene tar or isoprene tar. Chlorinated acetylene tar is much more irritating than the original tar, but the carcinogenic power has been greatly reduced. Other irritants which have given negative results on the mouse's skin are acridine, the effect of which on the human skin is well known, and the product obtained by combining ethylene with phenyl magnesium bromide in the presence of nickel chloride. Other instances could be taken from the industrial diseases of the human skin.

It seems, then, that the irritation which causes the growth of cancer must be of a special kind or must act upon some special element in the tissues.

2. No invariable relation has been found between the temperatures at which these various materials are formed and their comparative activity in producing tumours. Thus the three tars made from coal at 450°, 560°, and 1,250° show an ascending order of potency; the 820° isoprene tar is more active than the 700° tar; and experiments still in progress indicate that the 920° skin tar will prove more effectual than the 780° tar. In all these cases the higher temperature gives the more active product. But, on the other hand, in the case of acetylene, a low temperature (700°) gives a product more active than that produced at 800° to 900°; possibly at the latter temperature a larger proportion of the gas is decomposed into carbon and hydrogen. The errors inherent both in the measurement of the temperature to which the substance is exposed, and in the comparison of different series of mice, take effect upon such experiments, and no general conclusion on the influence of temperature can be drawn at present. But the three sets of observations on products from (1) Durham Holmside coal, (2) Californian petroleum, and (3) isoprene, agree in indicating the importance of that range of temperature where the formation of aromatic compounds is known to be in progress.⁸

3. The idea suggests itself that acetylene may be a decomposition product common to coal, mineral oils, yeast, skin, and isoprene, and that the cancer-producing material obtained from these very diverse sources is actually formed from acetylene by the synthetic process discovered by Berthelot. One would, then, expect a tar made from acetylene itself to be more active than any other, but this has not proved to be the case. Probably the yield of cancer-producing substance depends largely upon catalytic reactions which cannot as yet be controlled. Further experiments upon products from other tissues and tissue constituents are in progress in this Institute.

SUMMARY.

1. Cancer has been produced in mice by substances obtained by heating (1) acetylene, (2) isoprene, (3) yeast, (4) human skin, to temperatures ranging from 700° to 920°. Acetylene is the simplest organic compound from which a carcinogenic material has so far been obtained.

2. A petroleum which produced no tumours of any kind in mice in a prolonged experiment showed very active

cancer-producing properties after exposure to a temperature of 880°.

3. The cancer-producing substance present in coal tar is formed only in very small amounts below 450°; at 560° it appears in much larger quantity, and this increase continues at a slower rate up to 1,250°.

I wish to express my thanks to the staff of H.M. Fuel Research Station for their kindness in supplying the series of coal tars made at known temperatures.

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Remarks

ON

A NEW FLOCCULATION REACTION FOR THE SERO-DIAGNOSIS OF MALIGNANT DISEASE.

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INTRODUCTION.

THE value of accurate diagnosis of malignant disease, especially in early or latent cases, by a serological method does not require emphasis. Many attempts by various methods have been directed to this end, hitherto without conspicuous success, although the occurrence of profound changes, such as anaemia and cachexia, in the later stages of malignant disease would suggest the possibility of accompanying or antecedent changes in the serum.

Murray¹ has recently brought forward some evidence that a neoplastic reaction evoked at one site in an animal confers some resistance to such a change at another site, and he suggests that there is some systemic constitutional change. If such be the case, then it is not improbable that the medium of distribution of the locally produced change is the blood. On these grounds a serological reaction would appear *a priori* to be practicable.

In experiments carried out by me, it was found that extracts of cancer tissue used as antigen in complement fixation experiments often showed marked anti-complementary properties. It seemed possible that a method, in the nature of a colloidal flocculation reaction, which dispenses with the haemolytic system, might prove of value. Moreover, the fact that the Sachs-Georgi antigen employed in Professor Hirschfeld's laboratory, Warsaw,² gave a number of positive reactions with serums from cases of carcinoma, seemed to indicate the possibility of obtaining a specific flocculation reaction for malignant disease.

HISTORICAL.

The complement fixation test has been largely used to discover specific cancer antibodies in the serum. This method was first tested by Sampietro and Tesa in 1908,³ and they were followed by numerous other investigators, some with favourable and others with indefinite results. Weinberg and Mello⁴ obtained only twenty positive results from the serums of 100 cases of cancer. Subsequent observers also found the reaction to be without value. In 1912 von Dungern⁵ published the results of his method, for which he claimed accuracy in 100 per cent. of cases. Later observers have failed to substantiate his claims.

Other reactions have been described, such as the precipitin reaction, used by Engel in 1904, and the anaphylactic methods examined by Pfeiffer and Finsterlin.⁶ The haemolytic properties of cancer serum were noted by Weil⁷ and further examined by Crile.⁸ On this property was based the cuti-reaction of Elsberg.⁹

The antitryptic reaction described by Marcus¹⁰ was more fully examined by Brieger and Trebing.¹¹ Other reactions which may be mentioned are the meiotagmin reaction of Ascoli and Izar,¹² the ferment reaction of Abderhalden,¹³ the fallacies of which were pointed out by Leitch,¹⁴ and the "epiphanin" reaction of Weichardt.¹⁵ Freund and Kaminer¹⁶ have published a turbidity test based on the theory of a destructive action of normal serum on cancer cells. Shaw-Mackenzie¹⁷ describes a similar turbidity test for carcinoma, using saponified extracts of cancer tissue.

A chemical precipitation reaction is described by Kahn,¹⁸ to which class Botelho's reaction also belongs.¹⁹ The latter has been shown by me to have little diagnostic value.²⁰ The rate of sedimentation of red corpuscles has been used for the diagnosis of cancer by Hoffgaard.²¹

The flocculation method was first used by Weil and Braun.²² They noted the flocculating power of cancer serums upon lecithin solution in 17 cases. During the course of this work Roffo²³ has published a flocculation reaction for the diagnosis of malignant tumours based upon the Sachs-Georgi reaction, but the details of his method are not very definite.

METHODS.

1. Antigen.

In the endeavour to find a satisfactory antigen over two hundred preliminary experiments were carried out with cancer serums, using different extracts of tumour tissue. None of these antigens nor various combinations of them proved consistently satisfactory. No better results were given by a "polyvalent" antigen of several carcinomatous tissues, an ether extract after the method of Shaw-Mackenzie, or a saline extract. The following method has been finally adopted, and has proved in general satisfactory.

Method of Preparation.

Breast carcinomata, preferably of a rapidly growing cellular rather than of a scirrhous type, have been the tissues selected. The breast is received sterile from the operating theatre. A small portion is taken for microscopical examination. The breast is then frozen in the refrigerator at about -6° C. for three to five days. The growth can then be readily dissected out and cleaned from the hard surrounding fatty tissue.

The tissue is minced fine, with sterile precautions, and successive portions are frozen with liquid oxygen and pounded to a fine state of division in a sterile mortar while so frozen. This step is not essential. Carcinomatous tissues minced very finely (by means of a mincer made by the Laboratory Supply Association) and treated as described below have recently given equally satisfactory antigens. The tissue is weighed (for example, 10 grams) and double the weight of sterile saline is added (for example, 20 c.cm.) and the whole shaken and allowed to extract in the ice-chest overnight. The tissue extract is centrifuged down and the supernatant fluid pipetted off. The same volume of sterile saline is again added, and the procedure repeated four times till the supernatant fluid is free from colour. The saline extraction extends over a period of five days. The saline extracts are rejected and the washed tumour tissue is dried overnight at 37° C. on a porcelain dish. When dry the tissue is weighed, and of acetone five times the weight is added to the tissue, which is extracted in a well stoppered glass bottle in the cold incubator at 18 to 20° C. for seven days, with frequent shaking. At the end of this period the acetone is filtered off through filter-paper (first acetone extract). Half the original bulk of acetone is added and the extract replaced at 18 to 20° C. for twenty-four hours. The acetone is again filtered off (second acetone extract). These acetone extracts are rejected. The remaining tumour tissue is kept at 18° C. till dry, or at 37° C. for two hours, and ground up. Absolute alcohol equal to five times the weight of dried tissue is added. The tissue is extracted at 18 to 20° C. for ten days. The alcohol is filtered off through filter-paper into an amber-coloured glass-stoppered bottle. This forms the stock alcoholic antigen, which contains the saline-insoluble alcohol-soluble acetone-insoluble substances of the tumour tissue.

According to the strength of the antigen, the stock solution is diluted with absolute alcohol. This amount is determined for each antigen by finding the dilution which gives the maximum flocculation with positive serums. This appears usually to be 1 in 4, but varies considerably with different extracts.

2. Solutions Required.

(a) A 1 per cent. alcoholic solution of cholesterol is prepared by dissolving 1 gram of cholesterol (Kahlbaum) in 100 c.cm. of absolute alcohol in a glass-stoppered bottle.

(b) Sterile normal (0.85 per cent.) saline. The solution is made with distilled water, and should be free from calcium.

3. Apparatus.

(a) The trays, flocculation tubes, and pipettes are those used in the Sigma reaction.²¹ They are sterilized by dry heat.

(b) The dropping apparatus for preparing the saline emulsion of antigen is shown in the drawing (Fig. 1). It consists of a Winchester flask, tightly corked with a large rubber stopper, through which pass: (1) The delivery tube, to the outer end of which the end of a Dreyer-Ward dropping pipette is attached; the inner end is drawn out and lies below the level of the saline

FIG. 1.—Dropping apparatus for preparing saline emulsion of antigen.