GROWTH INHIBITION BY SUBSTANCES IN LIVER*

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It appears from the literature that a number of inhibitors of growth *in vitro* have been described. The present work is concerned with attempts to isolate inhibitory principles from liver, a preliminary report of which has been published previously (1).

Baker and Carrel (2) have demonstrated that the lipoidal (ether-soluble) fraction of serum contains an inhibitor of growth, and we have found some inhibitory activity in ether-soluble fractions of liver (1). The chief inhibitory principles in liver have, however, been extracted by aqueous or alcoholic media, as recorded by Walton (3) and by Heaton (4). Heaton's work has been continued by Medawar (5) who has extracted from malt, and from various other vegetable materials, an inhibitor of similar nature to that present in liver. This material, it is noted, is highly specific for mesenchyme, as was the case with some of our own preparations (1). Shibuya (6) has developed an extract of liver apparently in the aqueous fraction but "in close combination with lipoids" which inhibits fibroblasts *in vitro*, and he claims that it causes experimental tuberculous lesions *in vivo* to become walled off through development of mature connective tissue in involved areas.

The inhibitor described by Simms and Stillman (7) is different from the foregoing in that it is precipitated from aqueous solution by alcohol. This material is believed to be present in adult tissue and is released from it by tryptic digestion. These workers have shown that whey produces similar effects. Another alcohol-precipitated inhibitor from urine has been described by Rohdenburg and Nagy (8) as effective in slowing the growth of Colpidium, although we have observed that this has no retarding effect upon chick tissues *in vitro*.

Certain substances of known chemical nature have been found to be inhibitory to growth. Katzenstein and Knake (9) have noted that a number of substances which are active in reducing surface tension, including bile salts, organic acids, and alcohols, are inhibitory to fibroblasts but not to epithelium. They observe that this effect is produced by amounts of these various substances which produce about an equal degree of surface tension lowering. Roffo (10) has found that irradiation of lecithin solutions causes them to become inhibitory. Mayer (11) reports that a kephalin extracted from

brain is inhibitory to tissue cultures but only when in the presence of growth-promoting extracts.

Vogelaar and Ehrlichman (12) have found the reverse to be true of glycine: its inhibitory effect is considerably decreased when serum is added to the medium.

Haddow (13) has shown that certain carcinogenic hydrocarbons inhibit growth *in vivo* and the observations of Earle and Voegtlin (14) show that inhibition *in vitro* occurs, but only after a long period of time. Many other inhibitors of experimental tumors *in vivo* have been described. Murphy and Sturm (15) have described factors in mouse placenta and embryo skin which inhibit the growth of mouse carcinoma. A partial review of other tumor inhibitors is given by Simms and Stillman (7). It is hardly necessary to emphasize the importance of growth inhibition to the tumor problem. Murphy (16) has made the suggestion that neoplasia represents a disturbance of equilibrium between inhibitors and accelerators of growth, an hypothesis which appears to us to have great merit.

There are two main reasons why it seems appropriate to use the tissue culture as a test object in this study of growth inhibitors. In the first place, growth of cells in culture can be made to take place at a maximal rate, as if those influences which keep growth in check in the living animal become ineffective after explantation. Thus, the tissue culture would seem to be a *carte blanche* upon which the action of inhibiting substances would be obvious and unequivocal. In the second place, any fundamental differences in reactivity of normal and malignant tissues can easily be discovered, since the two types of tissue grow with comparable rates under *in vitro* conditions, and since it is easy to maintain equivalent conditions of nutrition and of concentration of inhibitors for different tissues which is clearly impossible *in vivo*.

The main difficulty in interpreting results, in such work as this, depends upon the fact that growth inhibition must be at all times distinguished from "toxic" effects on cells. This is especially important since, in separating various factors from tissue, it is at times necessary to use unphysiological reagents. Many workers have realized this, and Medawar (5) has drawn up a table summarizing the distinctions between lethal factors and one fairly well defined growth-inhibiting factor.

In the absence *a priori* of any definitive distinction, it seems well to state what we hope to find: namely, substances which are present in tissue (or may easily be obtained through extraction of tissue constituents) in concentrations approaching those which are inhibitory to the growth of tissues *in vitro*. We expect that such an inhibitor, derived from tissue, retards growth over a wide range of graded concentrations compatible with cell life; that growth continues to take place at the retarded rate, and

increases to a rate comparable with that of untreated tissue after removal of the inhibitor. A substance complying with this definition may be considered potentially important in the physiological regulation of growth.

Methods

Tissue Culture.—Routine assay of substances was performed by the use of hanging drop tissue cultures, as described in an earlier paper (1). Each group of cultures was controlled by a series of tissues grown in Tyrode solution with the addition of one part in six of heparinized chicken plasma. Embryo tissues, explanted in this medium, clotted it readily; in the case of mouse sarcoma and carcinoma it was usually necessary to add a trace of embryo extract (about 1:20). Substances being tested were made up in Tyrode solution and plasma was added. Cultures were examined daily and growth measured on the 3rd day, with the aid of a camera lucida. Measurements of the original explant were made in two diameters, and its "mean radius" calculated therefrom;

TABLE I

Calculation of Growth Quotients of Typical Inhibited Cultures

Medium	Average of five cultures		Growth quotient	Growth relative to control
	Mean radius of growth—3 days	Mean radius of explants		
				per cent
Tyrode	14.8	25.5	0.582	(100)
Extract 4 1:16	0		0	0
Extract 4 1:24	0.4	24.0	0.017	3
Extract 4 1:32	2.4	21.2	0.113	19

the width of the growth zone was then measured in four places and the mean value of these measurements was termed the "mean radius of growth." Typical cultures from all series have been fixed in Zenker's fluid and stained by hematoxylin. Each reported observation on inhibitory activity of an extract represents measurements on at least two series of five cultures each, for each dilution.

Calculation of Growth Rate.—The growth of each explant was expressed as: $\frac{\text{mean radius of growth}}{\text{mean radius of original explant}}$. The mean value of this growth quotient for each series of explants in a test solution was then calculated as per cent of the corresponding value for the explants in Tyrode solution. Thus, one experiment testing the activity of serial dilutions of an alcohol-soluble extract of rat liver showed the values given in Table I.

Duplicate series, each with five or more explants taken from the same organ, yield growth quotients which are consistent within 10 or 15 per cent, provided very large fragments of tissue are not used. There is great consistency in the daily radial growth rate during the first 4 days of culture.

Technique of Extraction of Inhibitors.—Saline extracts were made by adding three volumes of Tyrode solution to one of sterile liver pulp, made by passing liver tissue

through a press. The material was then shaken 15 minutes and cleared of solid particles by centrifugation. In subjecting these extracts to further procedures it was generally necessary to keep the material sterile. Alcoholic extraction was done similarly. Alcohol was removed by distillation *in vacuo* at room temperature, and isotonic solutions were then made by the addition of proper proportions of sterile Tyrode solution and distilled water. Saline extracts of adult rat, mouse, and fowl livers, and alcohol extracts of these and lamb, calf, and other livers have been used. It was found possible to sterilize most extracts by diluting them with alcohol to a final concentration of 70 per cent alcohol; after standing overnight the alcohol was distilled off *in vacuo*. Thus it was unnecessary to observe sterile precautions in the course of chemical procedures. A number of known heat-stable substances were sterilized by heating their solutions at 100°C. for 15 minutes.

Analyses of Media.—During the course of the work, numerous chemical analyses of growth media have been necessary, particularly in determining whether these media contained unphysiological or injurious amounts of various substances added in the course of their preparation. These determinations have in all cases been done by well known customary routine procedures. In most cases, we have done chloride analyses on media to establish their isotonicity. pH has been adjusted within the physiological range, using phenol red in a concentration of about 1:20,000. In many cases, whenever indicated, control cultures have been planted in media containing alcohol, acetone, potassium chloride, etc., to correspond with abnormal amounts of such substances in test solutions. Results showing inhibition have accordingly been accepted only when the media contained inert concentrations of reagents, *e.g.*, less than 1 per cent alcohol, 0.4 per cent KCl, or 0.01 per cent NH_4Cl.

RESULTS

It is important to realize that inhibitors are extracted from liver by both saline and alcoholic media, but that the inhibitory solutions obtained by these two methods differ considerably, and at times paradoxically, in their chemical behavior and physical properties. We shall, therefore, consider these two modes of extraction separately.

Saline Extraction.—Extraction of liver by Tyrode solution yields materials which are effective inhibitors of growth, especially of fibroblasts. The supernatant fluid after extracting liver with three volumes of Tyrode solution retards growth so that the growth radius is about 25 per cent of that of control cultures. This inhibition is partially lost in 1:2 dilutions of this extract, while growth is essentially normal in 1:4 dilutions. Subsequent reextraction of the pulp yields relatively less active material. Inhibitors in saline extracts are quite unstable. Thus, it is found that the inhibitory factors are partly precipitated from saline extract by the addition of alcohol, especially beyond 50 per cent concentration, and that they can be recovered from the precipitate under favorable conditions; that is, when the precipitate will return into aqueous solution. The addition of small amounts of acid (N/50 HCl or CO_2 gas) so as to bring the pH of the saline extract to 6.5 or 6.6, has a similar effect to the addition of alcohol: when the precipitate returns into solution it will produce inhibition. In one series of experiments, five portions of saline extract were acidified, and the five precipitates showed widely differing activities. These results appear in Table II *a*.

Those saline extracts which produce inhibition retain a heavy opalescence after long centrifugation at 3000 R.P.M. Loss of activity by addition of alcohol or acid is associated with precipitation and disappearance of this opalescence, and precipitates which show activity are always those which redissolve to make a solution of similar appearance. On the other hand, opalescence of the solution is not essential for inhibition since heating for a few minutes between 55° and 58°C. yields a heavy precipitate and leaves most of

TABLE II

*(a) Growth Inhibition by Acid Precipitates of Saline Liver Extracts**

Original saline extract Precipitates	Precipitation	Solubility of precipitate	Growth
	pH		*per cent*
			28
1	6.6	Partial	95
2	6.2	Complete	43
3	6.0	None	90
4	5.5	Almost complete	41
5	5.0	None	91

(b) Growth Inhibition by Heated Saline Extracts†

Original saline extract Heated	Growth
°C.	*per cent*
	32
50	50
58	57
75	64
75‡	93
100	92

* In these experiments, equal portions of saline extract (1 part of liver to 3 parts of Tyrode) were acidified by the slow addition of various amounts of N/50 HCl. The precipitates were redissolved to the original volume.

† Portions of saline extract of rat liver were heated for 30 minutes at various temperatures. The opalescent solutions became clear at 50°, but further coagulation of protein occurred at higher temperatures.

‡ Two portions of the same extract heated separately.

the inhibitory activity in clear solution. At higher temperatures, the inhibitors are lost in proportion to the volume of proteins coagulated (see Table II *b*).

Ultrafiltration of saline extracts through collodion membranes yields filtrates which are completely inactive (and in fact promote growth similarly to embryo extract), while full inhibition is found in the non-diffusible fraction.

These findings indicate that the inhibitors present in saline extracts are associated with colloids and in many cases at least are precipitated along with them.

Alcohol and Other Extracts.—Extraction of liver by 70 per cent alcohol yields about the same titer of inhibitory activity as saline extraction. In this case, however, the active material is freely diffusible, in general more stable, and at first sight appears to be quite different in nature; this is made apparent in Table III.

In view of these differences, it is of interest to know whether the two methods of extraction remove different groups of inhibitors, particularly since treatment of saline extracts by alcohol often results in their precipitation. We have found that following saline extraction of liver pulp, a further alcoholic extraction yields much less inhibitor, as in the case of a second saline extraction. Thus, removal of the colloidal inhibitors from liver by saline extraction leaves behind a residue which is correspondingly depleted

TABLE III

Properties of Liver Inhibitors

	Extraction by water or saline	Extraction by 50–100 per cent alcohol
Diffusibility through collodion membrane	None	Complete or nearly so
Stability at 50°C.	Fair	Complete
58°	"	"
75°	Slight	"
100°	None	"
Stability at pH 5.5	"	"
Adsorption to blood charcoal	Slight	Rapid

of diffusible, alcohol-extractable inhibitors. Moreover, the amounts of growth inhibitor obtained by saline and by alcoholic extraction of analogous parts of the same liver are approximately equal; in each case portions of the liver have been extracted by three parts by volume of Tyrode's solution or 70 per cent alcohol. These findings are given in Table IV.

It seems likely, therefore, that essentially the same agents are obtained from liver by saline or alcoholic extractions, but that in the saline extract they exist in a different state, intimately associated with proteins or other colloids.

Direct extraction of liver pulp by benzene, ether, and acetone has yielded much smaller quantities of inhibitory substances than alcohol, although ether extraction of saline extracts shows a slight degree of inhibitory activity in the ether-soluble portion.

Approximately equivalent amounts of inhibitory material have been obtained by aqueous or alcoholic extraction of adult livers, but embryo liver fails to yield activity (1).

Adsorbents.—The different state of the inhibitors in saline and alcoholic extracts is further shown by adsorption experiments.

Certain adsorbents, particularly Lloyd's reagent, can be shaken with saline extracts with the result that the inhibitors are removed without destroying the opalescence; in

TABLE IV

Growth Indices (Per Cent of Control) of Chick Embryo Tissue Grown in Extracts of Liver, Using Three Volumes of Saline or Alcohol to One of Liver

	Parallel saline and alcohol extracts of portions of the same liver	
	Growth in Tyrode extract	Growth in 70 per cent alcohol extract*
	per cent	*per cent*
Rat liver	25	40
Mouse liver	19	33
Fowl liver	16	11

	Successive extractions of the same liver pulp	
	Growth in Tyrode extract	Growth in second Tyrode extract
	per cent	*per cent*
Rat liver	33	71

	Growth in Tyrode extract	Growth in subsequent alcohol extracts*
	per cent	*per cent*
Rat liver	24	60
" "	22	101

* Alcohol was removed by evaporation *in vacuo*, following which an isotonic solution was made by addition of Tyrode solution to the original volume.

these instances the red color of hemoglobin and about two-thirds of the protein have also been removed from solution. Blood charcoal is less efficient in removing inhibitors from these extracts. Lloyd's reagent and charcoal actively remove from aqueous solution inhibitors which have been obtained by alcoholic extraction. The ready adsorbability of the diffusible, alcohol-extracted factors suggests that in saline extracts they may be held adsorbed to colloids. A few observations on serum containing a vital dye (T 1824) are worth mentioning because of the similar behavior of tissue inhibitors. This dye, when present in serum of animals after injection, is not dialyzable, and hence is probably in combination with serum protein. Precipitation of dye-containing serum protein by acid throws down the dye completely, while precipitation by alcohol leaves

80 per cent of the dye behind in the supernatant, whereupon the dye becomes dialyzable. The inhibitor in liver behaves similarly in the course of extraction (Table III).

Purification.—In a series of further fractionations, we first made use of a purified fraction of liver with hematopoietic activity (17). This was the product of alcohol elution from charcoal after adsorption of Cohn's fraction, G, which is a deproteinated, ether-insoluble, alcohol-precipitated fraction of liver. Following precipitation of this with ten volumes each of alcohol and ether, inhibitory activity was largely in the filtrate (extract 4 in Table V). In view of the alcohol solubility of active material, we have abandoned the alcohol precipitated fraction and used 95 per cent alcohol filtrate (Cohn's fraction F) in later stages of the work. Table V gives a summary of the active extracts obtained from this partially purified source of the inhibitor.

The alcohol filtrate (fraction F) was diluted to 10 cc. per 100 gm. of original liver and adsorbed by 1 gm. of charcoal. Nearly half of the total solids were removed. Preliminary elution of this charcoal with 30 per cent butyl alcohol (No. 81) removes much organic matter and some of the active material, but since the activity per milligram of total solids was small, we discarded this elute and eluted again with boiling 50 per cent ethyl alcohol for 15 minutes (No. 82). At this stage we found that the inhibitor in this solution can be adsorbed on charcoal in the presence of alcohol at room temperature and again eluted with hot alcohol (No. 85), which resulted in further purification but some loss in yield. This was concentrated to 1 cc. for each 100 gm. of liver originally used, and acidified to pH 3. 300 mg. of Reinecke's acid in hot water was added to 30 cc. of this, and the precipitate left in the cold room overnight. The precipitate was filtered and decomposed with silver. From this fraction considerable amounts of choline and nicotinic acid amide (inactive) were obtained and identified by gold salts and combustion. From the filtrate gold was removed and it was taken up in ethyl alcohol and precipitated with alcoholic picrolonic acid. Crystals were obtained and identified as ethanolamine picrolonate.

Ethanolamine was found to be inhibitory to growth in a concentration of approximately 0.5 mg. per cc. The activity of this and a large series of other amines have been studied and will be described in a future communication. The amines differ from the cruder extracts shown in Table V in two respects: their inhibitory action is dependent upon the pH of the medium, and is much greater on normal tissues than on tumors.

Pigments.—The relation between amount of yellow pigment and inhibitory activity is also shown in Table V. Purified extracts have been secured which contain only 5 to 10 per cent as much pigment as the original extracts per unit of inhibiting activity, but under these conditions we have been able to obtain only a very small yield of inhibitor from the original liver. Thus, although one colorless inhibitor (ethanolamine), and slightly colored crude

TABLE V

Activity of Various Fractions of Liver, in Terms of Dry Weight, Amount of Liver Extracted, Pigment, and Surface Action. (1 Inhibiting Unit Is Defined as the Amount Producing 75 Per Cent Inhibition of Growth When Dissolved in 1 Cc. of Medium)

Extract No.	Description	Amount of dry weight per inhibiting unit	Amount of liver yielding 1 unit	Relative amount of pigment in 1 unit*	Index of inhibitory power relative to surface activity†
		mg.	*gm.*		
Derived directly from liver					
A	70 per cent alcohol extract of liver	8	0.3	100	
Derived from fraction G of Cohn					
3	Alcohol-ether precipitate	8	14	81	0.1
4	Alcohol-ether soluble	6	1.5	22	1.3
6	Same, less $Ca(OH)_2$ precipitate	5	4.2	30	
14	Acetone precipitate of No. 4	35	25	64	0.06
68	No. 4, fraction not adsorbed on charcoal	5	3.3	19	0.5
67	Elution of No. 4 with cold alcohol	6	50	85	0.5
69	$HgCl_2$ precipitate	9	20	96	
74	Butyl alcohol extract of No. 4	2.4	4.2		
75	No. 74, ether-petroleum ether soluble	4.5	6	25	
78	No. 74, ether-petroleum ether precipitate	9	12	17	
79	Cuprous oxide precipitate of No. 4	2.0	16	6	
215	CCl_4 extract of No. 4	3	50		
Derived from fraction F of Cohn					
81	Elution from norite by butyl alcohol	9	3.1	62	0.7
82	Further elution with hot ethyl alcohol	3.4	6	10	2.6
84	No. 81, not precipitated by 20 volumes alcohol	5.3	3.1	9	
85	No. 82, adsorbed from cold alcohol and eluted by hot alcohol	1.5	6	6	
94	Reinecke salts of No. 85	1.5	25	0	
94*a*		0.5	100	0	
94*b*		1.2	33	0	

* Estimated photoelectrically, with the use of a 440 mμ light filter.

† Extracts (freed from alcohol) were diluted to produce a standard lowering of the surface tension of water (10 dynes per cm.). The index is expressed as the number of inhibitory units in 1 cc. of this solution.

fractions have been found, it is possible that pigmented inhibitors may have been lost in the course of purification. Medawar (5) has shown that the intensity of color of these pigments varies with pH and oxidation, but the great variations of color intensity per unit of inhibition shown in Table IV make it unlikely that the important inhibitors are pigments.

Surface Tension.—Surface activity likewise shows no correlation with inhibitory potency. Surface tensions of aqueous solutions have been determined with the Traube stalagmometer and the du Noüy tensiometer, and solutions of various extracts have been made up to produce a standard

TABLE VI

Concentrations of Various Substances Which Do Not Inhibit Tissue Culture Growth

Substance	Concentration
	mg. per cc.
Heparin (commercial)	5
"Inhibitor" from urine (8)	10
Potassium chloride	4
Glycine	10
Tryptophane	4
Phenylalanine	5
Tyrosine	5
Aspartic acid	5
Histidine	4
Arginine	2
Lysine	2
Thiochrome	1
Choline	5
Nicotinic acid amide	2
Vitamin B_1	8
B_6	Amount extracted from 100 gm. liver
B_7	Amount extracted from 100 gm. liver
Inorganic ash	Ashed inhibiting extract 6

lowering (10 dynes per cm.) as shown in Table V. It will be seen that there is little correlation between inhibition and surface activity in these materials. Two extracts in particular (3 and 14 in the table) show this degree of surface activity in concentrations which are wholly ineffective in influencing growth. A soap solution with surface tension equal to a completely inhibitory acetone-alcohol extract of liver had no influence on growth.

Known Constituents of Liver.—A number of known substances present in liver were tested on tissue cultures and found to be inactive in such concentration that they might be considered absolved of responsibility for the inhibitory action of liver preparations. The inactive concentrations of these substances and of certain others tested appear in Table VI.

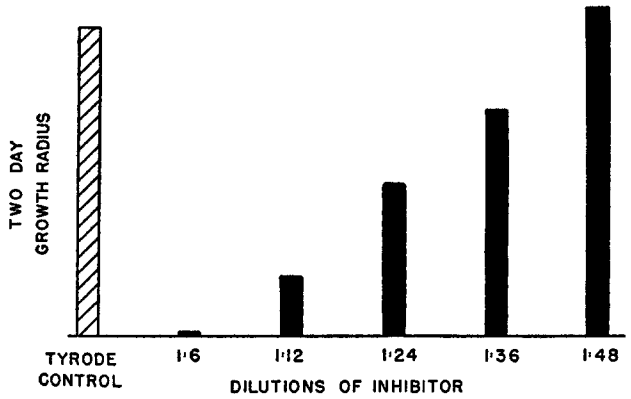
Influential Effects on Various Cell Types.—The large majority of active fractions contained some yellow pigment and were acid before neutralization for use (pH 4–5), and produced their most marked effects upon the growth of fibroblasts (Fig. 1). In these extracts, epithelium frequently showed growth in a concentration which completely inhibited fibroblasts, but generally failed to grow in twice this concentration. In no case were extremely luxuriant growths of epithelium seen, such as occurred in dilute solutions of bile, where the effect might reasonably be attributed to lowering of surface tension. Successive dilutions of inhibitory extracts showed a gradually decreasing inhibition, the margin between complete inhibition and normal growth covering a range of dilutions of about 1:8 (Text-fig. 1, Fig. 2).

Occasional mitoses were seen in fibroblast cultures inhibited as much as 80 per cent by these fractions, but these were much less frequent than in control cultures. Following 24 hours' treatment with colchicine, occasional mitoses (1 to 2 per cent) could be seen even in cultures the radial growth of which was completely arrested: control cultures with colchicine showed 50 to 60 per cent of the cells in mitosis. The cells in inhibited cultures tended to be smaller than normal. Fibroblasts frequently grew out in long, rope-like groups with two or three cells abreast containing numerous lipoid inclusions. In most cases the cells of inhibited cultures were somewhat more granular than in control cultures and the cell borders were seen much more distinctly under the low power. It was observed that inhibited cultures grew at a more or less constant slow rate but survived much longer than control cultures, reaching in 3 or 4 weeks a size comparable to that of a 3 day control culture (Text-fig. 2).

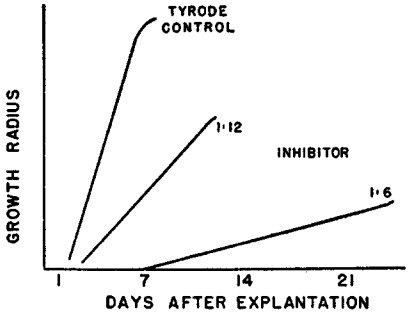
Cultures of sarcoma 180 in these extracts showed inhibition in the same concentration as did cultures of normal fibroblasts. The usually broad sarcomatous fibroblasts were relatively much smaller and narrower when inhibited (Fig. 3). Variation in pH had very little effect on the degree of inhibition of sarcomatous or normal cells. This is in direct contrast with the conditions in cultures grown in ethanolamine solutions, in which sarcoma cells were more resistant to inhibition than normal cells.

Growth Inhibition and Cell Function.—Evidence of undamaged cell function, given by normal pulsation of heart fragments, was commonly seen in concentrations of 4 or 5 inhibiting units per cc., about twice the amount necessary to prevent all outgrowth of cells. Concentrations of two extracts (4 and 85) containing 4 units per cc., had no effect on the rate of oxidation of glucose by slices of mouse liver and sarcoma (determined by Warburg's method). Concentrations of 8 units per cc. inhibited oxidation 50 per cent

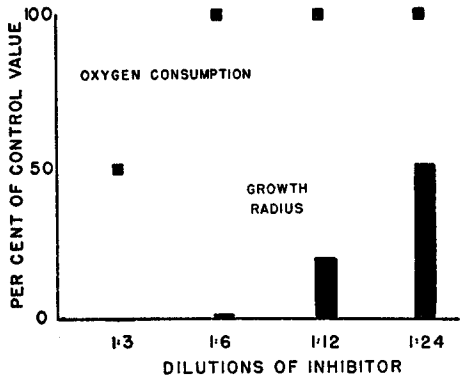
or more and produced pycnotic changes in the nuclei of cultures (Text-fig. 3). Goldfeder (18) has shown that in cultures, the growth of which is com-



TEXT-FIG. 1. Change in inhibitory potency with increase in concentration of inhibiting medium. Hanging drop cultures of chick embryo liver. Extract 6. 1:12 is equivalent to 1 inhibiting unit per cc., or 5 mg. of solid per cc.



TEXT-FIG. 2



TEXT-FIG. 3

TEXT-FIG. 2. Retardation of growth in inhibiting media. Explants of chick embryo heart in Carrel flasks. In this experiment, media were renewed twice weekly. Extract 85. 1:12 is equivalent to 1 inhibiting unit per cc., or 1.5 mg. of solid per cc.

TEXT-FIG. 3. Oxygen consumption of tissue in inhibiting media. Growth was measured in coverslip cultures of chick embryo liver; oxygen consumption of mouse liver in the same extracts was measured by Warburg's method. Extract 6, as in Text-fig. 1.

pletely inhibited by radium, there is likewise only partial inhibition of oxidation. Thus there is a wide margin between inhibiting amounts and those which produce visible damage to tissues, or which alter functions other than growth.

Reversibility of Action.—When inhibited cultures were washed free of the inhibiting solutions, growth recommenced and was frequently sudden in its onset, as noted by Medawar (5) (Fig. 4). When a washed, inhibited culture was treated with 25 per cent chick embryo extract, this increase in growth was especially explosive, and often extensive liquefaction around the explant followed within a few hours.

Inhibition in the Presence of Growth-Promoting Extracts.—Routine assay of extractives has, as described above, been done using solutions of these substances in Tyrode solution, and cultures in Tyrode solution alone have been used as controls. Under these conditions, the inhibition as measured represents a retardation of activity below the level of the "residual growth energy" of the explant. It must be remembered that in a plasma culture of which Tyrode solution is the main constituent, there can be little growth in the strict sense which implies building of new protoplasm, since the materials for such growth are few. We have, therefore, done parallel experiments in which the inhibiting and control media contain growth-promoting factors in the form of 25 per cent embryo extract or synthetic media containing peptone and added factors, as described by Baker (19). We have found uniformly that in the presence of these growth-promoting factors, retardation and complete inhibition of growth are brought about by the same concentrations of inhibitor as in the case of cultures lacking these factors. This is equally true where growth inhibition is obtained by the use of ethanolamine or by the cruder fractions of liver. Investigation of the carbohydrate and nitrogen metabolism of tissues cultured under these various conditions is now under way.

In Vivo Experiments.—It was possible to administer daily intraperitoneal injections of one extract (No. 85) to mice in amounts of 0.6 to 0.8 inhibiting units per gram of body weight. Death occurred after ten or twelve injections with signs of peritoneal irritation and ascites, and occasionally necrosis of cardiac muscle. In mice bearing sarcoma 180, some retardation of tumor growth was seen, probably due to inanition. No histological abnormalities in tumors were seen, in contrast to Heaton's findings (4).

DISCUSSION

The evidence presented indicates that there are at least two inhibitory constituents of liver having different biological properties. Their behavior shows that they may be of importance in regulating tissue growth, but their exact physiological significance must be discovered by future work. One of these inhibitors is ethanolamine. This substance, which has been definitely identified as an active constituent of some of the inhibitory extracts,

has properties in common with many related compounds. One of these, amino ethyl phosphoric acid, has been identified in tumors (20) and in intestine (21). The observation by Mayer (11) that kephalin is inhibitory only in the presence of tissue extracts raises the question whether, under these conditions, liberated ethanolamine may not be responsible for this activity.

The bulk of the inhibitory activity of liver extracts is not due, however, to ethanolamine or related compounds, since in all of the cruder extracts characteristics of ethanolamine (dependence upon pH, relative ineffectiveness in tumor cultures, and various morphological effects) are wholly masked. No other inhibitor has been identified chemically, and since the characteristics of inhibition by the various crude extracts are quite similar, it is possible that a single substance may be responsible for their activity.

The complex adsorptive behavior of these inhibitory materials is of interest. Although they are stable following a certain degree of purification, we have seen that they are relatively unstable in saline extracts and probably also in the state in which they occur in tissue. It is possible that this lability of tissue inhibitors, due to their close combination with colloids, may be useful in the regulation of cell growth.

It can also be seen that this behavior makes it difficult to distinguish, on a basis of simple reactions, between various unidentified principles. It is wholly possible, for example, that the alcohol-soluble materials described here are identical with the trypsin-digested inhibitor of Simms and Stillman (7), although the latter can be precipitated by alcohol. For we have presented evidence which strongly suggests that the inhibitors which alcohol precipitates with the colloids in saline extracts are in part identical with those which alcohol extracts from whole liver. Similarly, it is clear that the heat stability of a crude inhibitory extract depends upon the circumstances of its extraction.

The fact that these inhibitors have equivalent potency whether in a colloidal form or not, shows a contrast between their action and that of glycine, since Vogelaar and Ehrlichman (12) have shown that tissues are protected from the inhibitory action of that amino acid in the presence of serum.

SUMMARY

Certain tissue constituents inhibitory to cell growth, extracted from liver, are described. The findings indicate that inhibitory material is adsorbed to colloids in the native state and is freed from them by alcohol extraction. One inhibitor, ethanolamine, has been isolated. This substance differs in its biological properties from the bulk of the inhibitory material present

in liver. Progress in purification of other inhibitors is described, and it is shown that inhibition by these extracts is not correlated with surface activity or with the presence of pigmented constituents.

The inhibitors have common properties which suggest that they are of physiological significance in the regulation of growth: action over a wide range of concentrations at which other cell functions are undamaged; reversibility of action; presence in adult liver in concentrations near those which inhibit growth *in vitro*, while in embryo liver they are found only in much lower concentrations.

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EXPLANATION OF PLATES

PLATE 20

FIG. 1. Morphology of growth in culture of chick heart in 1 inhibiting unit per cc. Note mitosis: they are occasionally seen in inhibited cultures. 3 day culture, extract 13.

FIG. 2. Growth of chick heart in successive dilutions of inhibitory extract 6. Hanging drop cultures of chick embryo heart, 48 hours after explanation.

(*a*) 2 units per cc.

(*b*) 1 unit per cc.

(*c*) 0.75 unit per cc.

(*d*) 0.5 unit per cc.

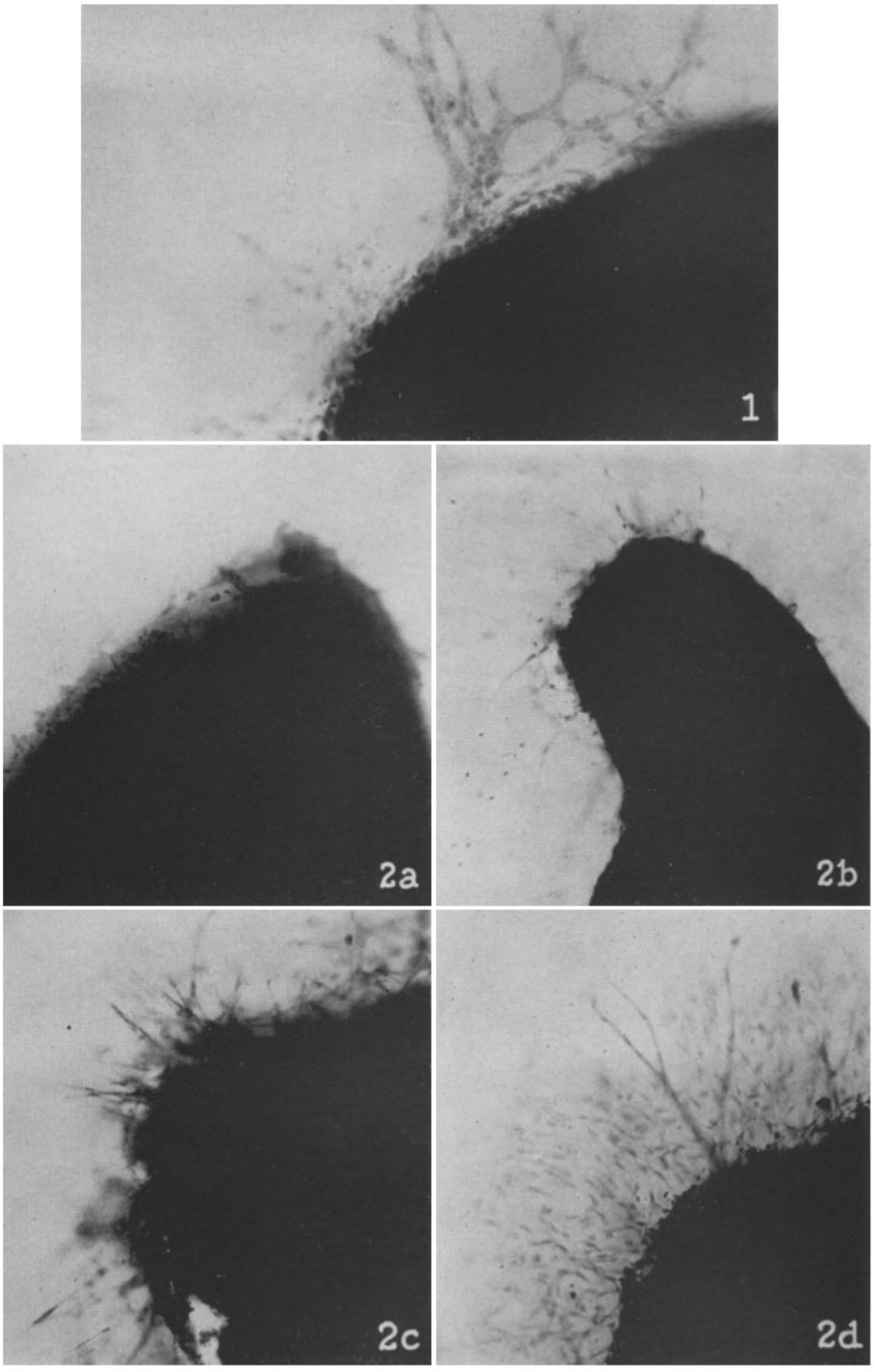

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PLATE 21

FIG. 3. Inhibition of sarcoma 180.

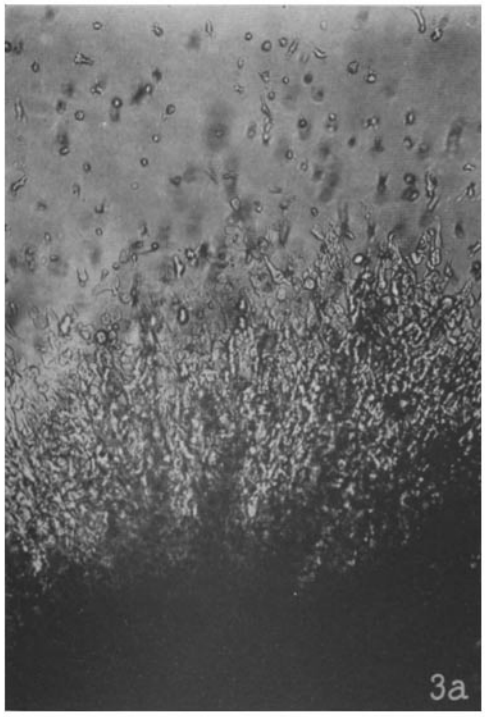
(*a*) Control culture (2 days).

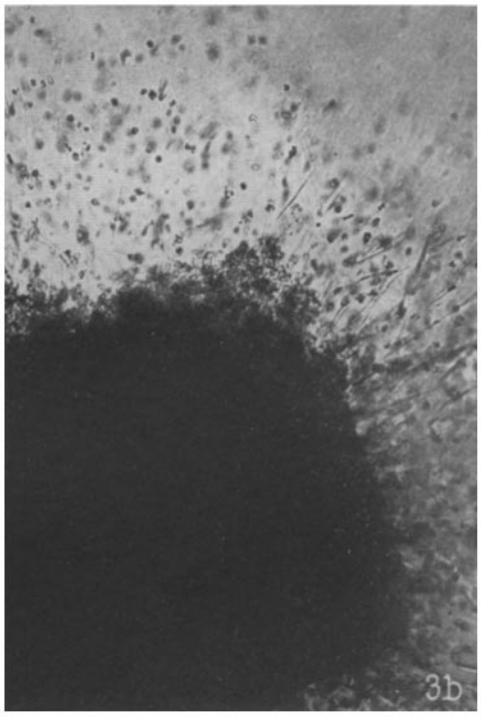
(*b*) Culture in alcoholic extract of calf liver.

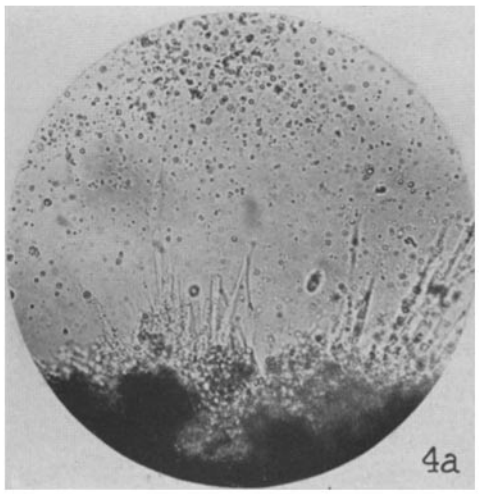
FIG. 4. Recovery from growth inhibition. Culture which showed no growth after 3 days in extract 82, 2 units per cc.

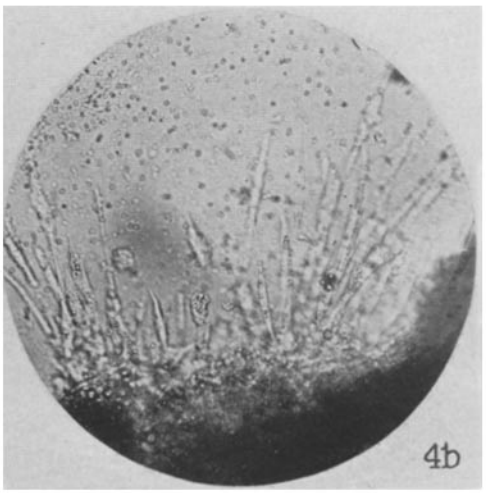
(*a*) 18 hours after washing off the inhibitory extract.

(*b*) 24 " " " " " " "









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