A METHOD FOR THE STUDY OF INDUCED INTERFERENCE WITH TRANSPLANTABLE TISSUE GROWTH

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The methods to be described make possible a statistical decision on the induction of inhibition and of acceleration of tissue growth. The major laws of transplantability govern tumors and normal tissues indiscriminately (1). For this reason, the methods may have an usefulness in the study of transplantable tissues beyond that already observed for transplantable tumors.

The use of transplantable animal tumors as test material has limitations due to a number of variables encountered, which cannot now be directly controlled. Therefore, proof of experimental interference with tumor growth requires large numbers of tests and must be judged statistically. The two main variables are the inherent growth energy of the tumor used for inoculation and the susceptibility of the inoculated strain of animals to the growth of the particular tumor. In other words, the size attained in a given time by an inoculated tumor transplant depends on host susceptibility and tumor growth energy. When tumor transplantation is employed to evaluate the influence of tissue extracts or their derivatives on growth another group of variables is met, which pertains to preparation and preservation of the agent.

The effectiveness with which host susceptibility and tumor growth energy are controlled is the basis for estimating experimental interference with tumor growth. The use of inbred, relatively pure line stocks of animals will reduce somewhat the variation of host susceptibility. The influence of this variation may be reduced by the system used in this laboratory of having a control transplant and a test transplant in each animal. In the absence of independent estimates of host susceptibility and tumor growth energy the most convenient control of these variables is afforded by planning experiments so that their influence will be distributed at random. The application of the principle of randomization (2) in the experimental design and procedure has led to consistently reproducible results from relatively small samples of cases.

The basis for the method employed in this paper is the inoculation of both a control graft and a test graft in the same animal. This is a routine procedure in this laboratory and is based on the assumption that variation due to host susceptibility can thus be eliminated or minimized. In other words two grafts from the same tumor, implanted in one animal, ought to grow at about the same average rate. For convenience the agreement between growth rates will be called matchableness. Experience with different strains of mice and different kinds of tumor revealed that the degree of matchableness varied. Therefore some measure of the matchableness is required for valid interpretations of results based on treating one of the graft pairs: because the interpretation is going to depend on the observed difference in the growth rate of the test graft from that of the control graft, that is, on a change in matchableness. This paper sets forth a measure of matchableness and its use in evaluating results of this kind.

Experimental Design and Procedure

The exposition will be clearer from acquaintance with the following definitions of some frequently used terms.

1. A *test agent* is an agent whose influence on growth is the subject of investigation. The agents tested in this study are tissue extracts or their derivatives.

2. The control substance is either 0.9 per cent sodium chloride, Ringer's solution, or Tyrode's solution.

3. *Test tumor* refers to a tumor arising from an inoculum which has been exposed to the test agent before inoculation.

4. *Control tumor* refers to a tumor growing from a sample of the same tumor substrate as that used for the test transplant, but exposed to the control solution for the same length of time and under the same environmental conditions.

5. A test series is a series of animals each of which has been inoculated with a test transplant and a control transplant.

6. A control series is a series of animals each of which has been inoculated with two control transplants but no test transplants.

The experimental unit consists of four divisions each distinguished by a different substrate tumor of the same kind. The order in which the animals are inoculated is shown, horizontally, by cages in Fig. 1. This is a sample design applied to four test substances a, b, c, d, to be compared with a control solution e, using four tumor substrates and five cages of ten mice from the same stock. In the first division of this experimental unit, twenty grafts are cut from tumor substrate A to be approximately the same size, and are separated from cystic fluid and necrotic material. From this assortment grafts are selected at random and distributed so that there are two in each of the four test solutions and twelve in the control solution. After submerging, the grafts are flattened by a single squeeze with forceps to increase the area of exposure to the fluid. They are left in the liquids for 20 minutes at room temperature and then loaded into trochars preparatory to inoculation. A mouse from cage I is selected at random, marked for identification, and inoculated in the right groin with a graft from test solution a and in

the left groin with one from the control solution. The next mouse is selected at random from cage II, marked, and receives a graft from test solution b and one from the control solution. This is continued until a mouse from each of the five cages has received a control graft and a different test graft. At this stage the sequence returns to cage I, but instead of a graft from solution a, a graft from solution b is inoculated, and the sequence continues again to cage V. At this point the first substrate is discarded. The procedure in the other three divisions is similar, except that on the fifth return to cage I, one-half the experiment having been completed, the sequence of inoculation is reversed.

The make-up of each of the five cages can be seen from Fig. 1 to give each agent an equal number of representatives in each cage and from each substrate tumor. After

FIG. 1

The Design of an Experimental Unit

Mouse	Cage I	II	III	IV	V	Tumor substrate
1	a	b	c	d	e	A
2	b	c	d	e	a	
3	c	d	e	a	b	
4	d	e	a	b	c	B
5	e	a	b	c	d	
6	e	d	c	b	a	C
7	d	c	b	a	e	
8	c	b	a	e	d	
9	b	a	e	d	c	D
10	a	e	d	c	b	

Fisher prefers the Latin square to the systematic diagonal square because of possible diagonal ridges of influence. In agricultural problems these may occur because the experimental soil is continuous. Continuity of this kind can scarcely be disturbing in animal experiments where the "ridge" crosses several cages. The systematic design proposed in this paper is less confusing at the time of inoculation.

inoculation the mice are kept at room temperature under optimum laboratory conditions until the time for best judgement of the fate of the inoculated transplants. If too long a period is allowed some of the animals will show ulceration and infection of tumors and some will have died. In consideration of these factors and the maximum size of tumors for comparison, the optimum time at which to judge results is 21 days for Bashford Adenocarcinoma C 63, in the Rockefeller Institute strain of mice, whereas tumors of the same line in the Little albino A strain are measured at 24 to 26 days. For S 180 in Rockefeller Institute mice the optimum final time of measurement is 16 to 18 days. These are points which would have to be determined for different tumors in standard strains of mice.

The tumors are removed from the mice at the optimum time, separated from surrounding tissue and weighed. When a tumor is cystic and ruptures during removal, the weight of the escaped fluid is estimated from the increased weight of a piece of gauze

used to catch the fluid. When all the tumors in an experimental unit have been weighed and the results are recorded in terms of marks of mouse identification by cages, each case is assigned to the proper test agent and the influence of the different agents analyzed.[1]

When the experimental unit is repeated the positions of the test and control grafts are reversed in each alternate unit. The assumption underlying the procedure and design is this: repetition of the experimental unit, in which the influences of host susceptibility and of tumor growth energy are randomized, will lead to equal representation of each agent with respect to these two main variables in so far as they affect the growth rate of tumors. To what extent this assumption is tenable can be checked when the repetition makes at least fifty cases for each agent available for analysis.

System of Analysis

The methods of evaluating the influence of test agents on the growth of transplantable tissue will be illustrated by results obtained from a study of transplantable mouse tumors.

An agent (applied to grafts before inoculation) can inhibit, accelerate, or not affect the subsequent growth of the grafts. In the discussion of the analytical problems tumor growth is increase of tumor weight with time after inoculation.

The qualitative characteristics of tumor growth, in the gross, such as the degree of necrosis and cyst formation, might be so strikingly disturbed by test action as to invalidate the comparison of test tumors with control tumors by weight alone. This possibility was tested for Bashford Carcinoma C 63, growing in the Rockefeller Institute strain of mice. A control series of tumors (having a control side and test side tumor in each mouse), not ulcerated, of different sizes, taken from mice at different times after inoculation, were weighed in the gross and again after removal of cystic fluid and necrotic material. Table I is a summary of the actual quantities and of the proportions of macroscopically healthy tumor tissue.

The values in Table I for the means and standard deviations show these data to be representative of variation in tumor structure in the sense that they include all degrees of tumor structure from the almost completely necrotic to the almost completely healthy. Furthermore, these data show the proportion of healthy tumor tissue to necrotic tissue to be a major qualitative character of this transplantable tumor: necrotic material is present in every transplant and on the average is about one-half the tumor weight.

[1] By doing this and by the nature of the design of experiments subconscious bias is wholly avoided. Bias in regard to a test action is always a possibility in a prevalent design of experiments having all one cage of mice represent all cases of one test agent, and having the test tumors in that cage always in the same position in the mice.

The matchableness of tumor pairs in regard to qualitative characters is shown in Table II by the degree of correlation between the right and left groins in regard to proportion of healthy tumor tissue and to the quantities of healthy tumor tissue. It may be concluded that tumors paired in one mouse have proportions of healthy tumor tissue which are much the same even though the actual quantities are dissimilar. The high degree of correlation for proportions of healthy tissue together with the lower degree of correlation for actual quantities suggests that tumor structure is inde-

TABLE I

Statistical Description of Data Used for the Determination of Correlation Coefficients

	Control side tumors	Test side tumors
Number	69	69
	Per cent of healthy tumor tissue	
	per cent	*per cent*
Arithmetic mean	54.99 ± 1.43	52.16 ± 1.42
Standard deviation	17.26 ± 1.00	17.19 ± 1.00
Coefficient of variation	31.6	33.0
	Quantity of healthy tumor tissue	
	gm.	*gm.*
Arithmetic mean	1.29 ± 0.097	1.17 ± 0.076
Standard deviation	1.19 ± 0.069	0.94 ± 0.054
Coefficient of variation	92.3	80.3

Bashford Carcinoma 63 in the Rockefeller Institute strain.
All statistical constants qualified by probable error.

pendent of tumor size. Because the tumors were paired in each mouse the related sizes of the right and left groin tumors represent relative growth rates and it is therefore suggested that tumor structure is independent of tumor growth rate as well as tumor size, or that there is a more important factor than either of these. A more important factor would appear to be a systemic influence of the host, since the proportions of healthy tissue are more alike in tumors in one mouse than from mouse to mouse.[2] The conclusion follows that appraisal of the direct effect of an agent on tumor

[2] Though the correlation coefficient, r_c, appears to be significant in relation to the probable error, it should be more properly compared with the correlation coefficient for quantity with its probable error. The apparent significance may be due to the method of correlation, noted under Table II, or to the fact that some general uniformity may be expected from a control series of cases.

tissue by comparison of test tumors with control tumors by weight alone is valid so long as the transplants be paired, in the same relative positions, in one host and if a test agent be applied to grafts only before inoculation and is introduced in negligible quantity into the host. How little of a test agent will affect the host under these conditions can be determined from an analysis like that in Table III.

Another precautionary analysis concerns the variation of control tumor size. A significant difference in this variation between two series of cases precludes the comparison of the case results of the two series. In other words, unless the mechanism of tumor growth be known, which is not now the case, two series of cases having different levels of tumor growth rate are

TABLE II

Correlation of the Quantity of Healthy Tumor Tissue in Control Tumors with That in Test Tumors

	Correlation coefficients with probable errors	
	Proportion of healthy tumor tissue to total tumor mass in control tumor correlated with that in test tumor	Quantity of healthy tumor tissue in control tumor correlated with that in test tumor
Control and test tumors in same mouse	0.815 ± 0.027	0.622 ± 0.050
Control and test tumors in a different mouse*	0.387 ± 0.069	0.204 ± 0.078

Bashford Carcinoma 63 in the Rockefeller Institute strain of mice.

* Control tumor of one mouse compared with test tumor of the next mouse in order of necropsy.

not comparable. In Table III the form of this analysis is shown to be a comparison of the frequency distributions of control tumor weights, measured at the same time after inoculation, of three test series and one control series. The agreement between distributions was checked by the chi-square test (3).

The evident good agreement in Table III indicates that each agent was tested on adequately similar samples of tumor growth rate and so justifies the comparison, from series to series in one experiment, of case results. When the test series of two experiments have no agent in common but otherwise represent the same experimental material, the analysis shown in Table III is extended by a preliminary test of the agreement between frequency distributions in the two control series. The fact that the series in Table III represent different experiments (which had good agreement between control series) indicates the uniformity of variation of tumor growth rate at different times and with different samples of an experimental ma-

terial as variable as Bashford Carcinoma C 63, in the Rockefeller Institute strain of mice.

Fisher (2) in discussing the principle of randomization states: "Each pair of plants assigned their positions at random ensures that the estimates of error take proper care of all first causes of different growth rates, and relieves the experimenter from the anxiety of considering and estimating the magnitude of the innumerable causes by which his data may be disturbed." With respect to this assumption the analysis just outlined

TABLE III

Goodness of Fit of the Frequency Distributions of Weights of Control Tumors in Control and Test Series of Cases

Tumor weight	Series A	Series B	Series C	Control series	
				Left groin	Right groin
gm.					
0.1–0.4	27	22	12	23	22
0.5–0.8	17	13	7	13	13
0.9–1.2	7	12	5	7	8
1.3–1.6	8	11	8	4	4
1.7–2.0	7	8	8	4	5
2.1–2.4	3	3	3	5	5
2.5–2.8	4	5	4	5	4
2.9–3.2	4	5	3	3	0
3.3–3.6	4	2	4	3	3
Over 3.6	4	3	3	3	2
Total numbers.....	85	84	57	70	66
Chi-square.....	2.613	5.602	7.774	Standard	3.391
Probability*.....	0.975	0.778	0.606		0.933

Bashford Carcinoma 63 in the Rockefeller Institute strain of mice.

* Probability that deviations as great or greater could occur by chance alone in samples of the given size.

may be considered a check on the effectiveness of randomization in the particular experimental units, which are of limited size and composition due to practical considerations of time of each inoculation and of fatigue.

Analysis of test action requires both a test series and a control series of cases. There is some variability in size between two control tumors in the same relative positions in the same animal even with the most careful technique.

Characteristics of the initiation of transplant growth supply some reasons for this variability. The subcutaneous tissue of one groin may be less suited to tumor growth than that of the other groin. Each graft may have different amounts of vigorous cells. The host factor, which includes the

variation of blood supply and stroma formation, is stressed by the observations of Murphy (1) that the local lymphocyte response affects the take of grafts. The tumor factor is consistent with the fact that transplantable tumors are rarely collections of cells in the same state of growth (4). These factors and variability of graft size due to technique cause different growth rates for tumors paired in each mouse. In so doing they make analysis of test action more intricate. The matchableness of the paired tumors in each case is indicated by the ratio of the weights of the two tumors: in a series of cases it is shown by a frequency distribution of ratios, as in Table IV (a perfect match would be represented by the ratio 1.0). The ratio has a plus sign when the larger tumor of the pair is the control and has a minus

TABLE IV

Frequency Distribution of Ratios of Tumor Weights in a Control Series

Positive ratios	Over 10.5	9.5–10.4	8.5–9.4	7.5–8.4	6.5–7.4	5.5–6.4	4.5–5.4	3.5–4.4	2.5–3.4	1.5–2.4	1.4 to −1.4
Frequencies	8	0	1	0	1	1	2	1	2	11	32
Negative ratios	Over 10.5	9.5–10.4	8.5–9.4	7.5–8.4	6.5–7.4	5.5–6.4	4.5–5.4	3.5–4.4	2.5–3.4	1.5–2.4	
Frequencies	11	0	1	2	0	0	1	1	3	13	

Strain of mice, Rockefeller Institute.
Line of tumor, Bashford Carcinoma C 63.
Weight of larger tumor of pairs, 1.0 to 1.5 gm.
Number of cases, 91.

sign when the larger tumor is the test tumor.[3] The matchableness of a control series shown in Table IV and of a test series may then be compared by the chi-square test. If the fit is good, the conclusion follows that the test agent has not interfered with tumor growth. In this instance the more categories, so long as the number of cases in each is adequate, the more justifiable the conclusion. However, if the fit is poor the most rigorous evaluation of the test action so suggested is the goodness of fit in the number of categories which will each represent a distinct kind of growth effect, such as the inhibitory, the accelerative, and the ineffectual. The array in Table IV has been condensed in Table V into these three categories. A rule was adopted for this condensation to groupings of results with different kinds of tumors and different strains of mice. This rule is that when the frequency

[3] In describing a control series of cases, "test tumor" refers to an untreated tumor occupying the groin which the real test tumors in the same experimental unit occupy, right or left groin as the design may be.

distribution of ratios in a control series of cases fits a normal distribution[3] in many categories, it can be condensed to fit a normal distribution in three categories. In Table V, for those cases in which the larger tumor of each pair weighed 1.0 to 1.5 gm., for ratios taken to the nearest tenth, the best fit with a normal distribution occurs when the three categories are (*a*) +3.5 and over, (*b*) +3.5 to −3.5, (*c*) −3.5 and over.

A control distribution of this kind serves as a standard for comparison with a distribution of test cases in the same three categories. In this control distribution, condensation into three categories of a normal distribu-

TABLE V

Regrouping of Ratios of Tumor Weights of a Control Series in Three Categories as a Normal Frequency Distribution

	Apparent inhibition	No effect	Apparent acceleration
	Ratios over 3.5	3.5 to −3.5	Ratios over − 3.5
Observed grouped frequencies	14	61	16
Calculated normal frequencies	13.65	63.52	13.65
	χ^2 equals 0.513 n' equals 3 P equals 0.78		Normal frequencies calculated for a symmetrical curve using a limit of $\frac{X}{\sigma}$ equal to 3.09 in the "Table of deviates of the normal curve for each permille of frequency" (3)

Data as in Table IV.

tion took place at the ratio 3.5. A ratio with this characteristic will be called a grouping ratio. The numerical value of the grouping ratio is an index of matchableness of tumors paired in each mouse. It is a simpler and more wieldable description of this characteristic of the experimental material than an extended frequency distribution.

The ratio is preferred to the difference as the form of comparison in each case because it also expresses the proportion of healthy tissue whereas the difference in gross weights rarely expresses the difference in amounts of healthy tissue. Furthermore, most of the associations of tumor and host have a maximum latent period beyond which no transplant can be expected to start growing. This period is about 14 days for Bashford Carcinoma C 63, in the Rockefeller Institute strain of mice and about 20 days for that tumor in the Little albino A strain. The phenomenon has been demonstrated in rat tumors by Schrek (5). Hence, in cases of complete inhibition of tumor growth the increase of difference of control tumor weight from the weight of the test tumor, which is

zero, at any time after the maximum latent period may have been caused by the limitations of the host and not by the inhibiting agent, whereas the ratio, being infinity, indicates test action independently of the maximum latent period.

After the initiation of the transplant the tumors are parts of the host and therefore susceptible to metabolic changes like any other component tissue. The continuations of transplant growth in one host may reflect different local effects of metabolism and also of disease processes. Transplantable tumor growth is slower in sickly animals than in healthy ones. The variation of the grouping ratio with the growth rate in a control series of cases supplies an index of the influence on matchableness of factors affecting the continuation of transplant growth. The data in Tables IV and V are those cases in which the larger tumor of each pair was 1.0 to 1.5 gm. Similar arrays of this control series were determined and grouping ratios calculated for other weight ranges at the same time after inoculation. The results for Bashford Adenocarcinoma C 63, in Rockefeller Institute mice are presented as curves of grouping ratios for different sizes of the large tumor of each pair in Fig. 2 in comparison with those for S 180 in the same mouse strain and for the same kind of tumor in the Little albino A strain. There were at least fifty cases for each observed point in each curve and tumor weights were determined at the optimum time after inoculation for the particular strain of mice and kind of tumor. These curves describe the matchableness of tumor pairs, at the optimum time after inoculation, in a control series of each of the three different kinds of experimental material.

The particular usefulness of a standard curve of the kind given in Fig. 2 is the apportionment of cases, to three categories of a single frequency distribution, representing all tumor sizes. This apportionment is valid for any size of tumor and at any time after inoculation. Though the curves were obtained from results at the optimum time after inoculation they have been found to apply to any time after inoculation. The curves show that the weight of the larger tumor of a control series is a hyperbolic function of the grouping ratio. The general type of relationship is $y = \frac{c}{x - k}$ where y is the weight of the larger tumor, x is the grouping ratio and c and k are constants depending on the experimental material. In the case of Bashford carcinoma in Little albino A strain the relationship is given by

$$y = \frac{1.5}{x - 1}$$

This may be rearranged in the form

$$x = \frac{1.5 + y}{y}$$

Now, the grouping ratio is that standard ratio of larger tumor to smaller tumor in each pair such that a larger observed ratio indicates an inhibitory or accelerative effect. Therefore, for any given size of the larger tumor the hyperbolic curve may be used to compute the maximum size of the smaller tumor which will allow an interpretation of significant difference in growth rates of the two tumors. In other words $x = \frac{y}{w}$ where w is the standard weight of the smaller tumor such that any smaller observed weight of the

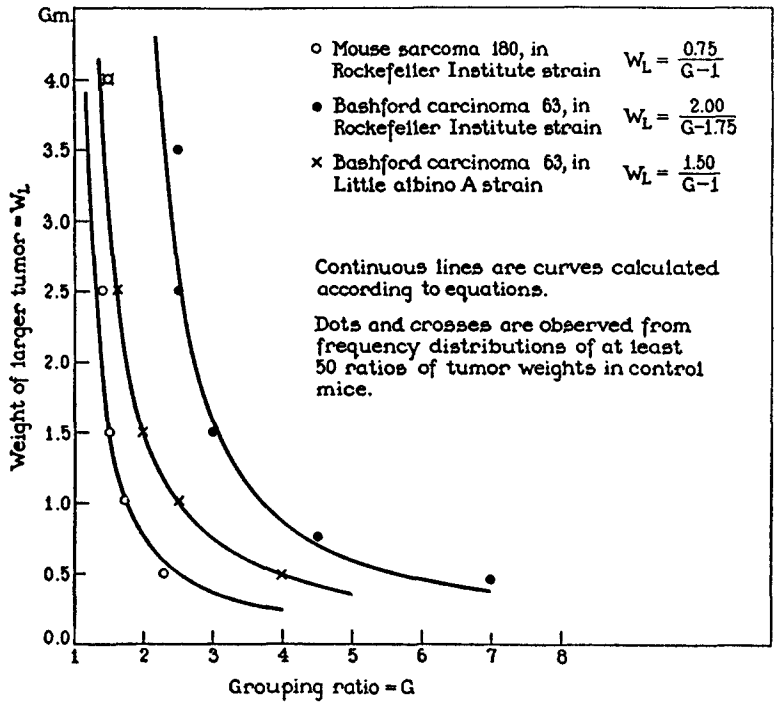

FIG. 2. Matchableness of transplantable tumors in different mouse strains as determined by the ratio of the weights of right and left groin tumor transplants.

smaller tumor indicates an inhibitory or accelerative effect, therefore the maximum size of the smaller tumor for this purpose is given by

$$w = \frac{y^2}{1.5 + y}$$

where w = standard weight of smaller tumor
y = observed weight of larger tumor

Therefore if W_o be the observed weight of the smaller tumor a case of inhibition of tumor growth is indicated when $W_o < W$ or $W_o < \frac{y^2}{1.5 + y}$ where y is the weight of the control tumor and W_o is the weight of the test tumor. A case of acceleration of tumor growth is indicated in the same way except that now y is the weight of the test tumor and W_o is the weight of the control tumor.

The two tumors in one case may have growth rates shown to be significantly different

by the foregoing analytical method. However, the main point of our experiments has been whether certain tissue extracts affected the growth rates of transplantable tumors. The point can be decided only by a comparison of a test series with a control series of cases and not by a single case since as we have already emphasized there are a number of other factors that may significantly change the growth rates of transplanted tumors.

TABLE VI

Reproducibility of Results

Set	Observer	Number of cases	Inhibitory		Ineffectual	Accelerative	
			Complete	Partial		Partial	Complete
a	S*	63	6	3	43	4	7
	M	77	11	4	49	3	10
b	S	52	20	8	18	3	3
	M	38	14	4	16	2	2
c	S	73	16	29	25	1	2
	M	77	18	25	32	1	1
d	S	22	1	1	7	9	4
	M	21	0	1	7	10	3

Goodness of Fit for Each Set of Results

Set	Five categories		Three categories	
	χ^2	P	χ^2	P
a	1.29	0.86	0.67	0.72
b	0.90	0.92	0.53	0.77
c	1.37	0.71†	1.00	0.61
d			0.23	0.83‡

Bashford Carcinoma 63 in the Rockefeller Institute strain of mice.

* The observers' names are abbreviated, S referring to Mr. Ernest Sturm and M to MacFadyen.

† Four categories, the two subcategories of "accelerative" cases were combined.

‡ Frequencies under "inhibitory" combined with "ineffectual."

By using curves of $w = \frac{y^2}{1.5 + y}$ it becomes a very simple matter to apportion cases to the three categories, inhibitory, ineffectual, accelerative.

The chi-square test may then be applied to the two distributions.

The application of this system of analysis to the design of experiments and technical procedure leads to consistently reproducible results with relatively small samples of cases. This claim is substantiated by the agreement between results, obtained by two observers using the same experimental material but a slightly different technique, shown in Table VI.

There are four groups of results in Table VI, each group representing one test agent. They were selected to illustrate reproducibility for different patterns of results obtained with the most variable experimental material in this laboratory. The observers' names are abbreviated, S and M. The categories of inhibiting action and accelerative action are broken down into two parts, one part to show absence of the test tumor and control tumor respectively, the other part to show the remainder of the cases in which both tumors grew. The numerical values of P,[4]—none under 0.61,—indicate that the results in each group were random samples of the same population of variables. In other words, the agent in each group was tested by the two observers on adequately similar samples of host susceptibility, tumor growth energy, and of the factors in the initiation and continuation of transplant growth.

SUMMARY

1. A design of experiments and technical procedure is described for the study of interference with transplantable tissue growth by test agents. The methods are guided by the principle of randomization and are based on the pairing of test and control in each host.

2. A system of analysis, employing well known statistical measures, is applied to results obtained with these methods and shows that they lead to consistently reproducible results.

3. A simple analytical method is proposed for correlating results obtained with different kinds of transplantable tissue in different strains of animals. This method is based on the condensation of an extended frequency distribution of events in control series of cases into a three category array of a normal frequency distribution. The three categories of test action are the inhibitory, the ineffectual, and the accelerative.

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[4] The accepted convention is that P, the probability that deviations as great or greater could occur by chance alone in samples of the given size, have a numerical value less than .05 for highly probable significance of the observed deviation and a value less than .01 for practical certainty of significance of the observed deviation.