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THE WALKER CARCINOMA 256 IN THE SCREENING OF TUMOUR INHIBITORS

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In the early stages of the examination of compounds as potential therapeutic agents for malignant disease it is customary to study their action upon malignant tumours, spontaneous or transplanted, in the smaller laboratory animals, rats or mice. Various tumours and conditions of test have been employed and various criteria of activity adopted. For the preliminary screening of compounds of several series we have used as test object the Walker carcinoma 256. The nature of the compounds and the results obtained with them have been the subject of a preliminary communication (Rose, Hendry, and Walpole, 1950) and will be published in greater detail in the near future; the object of the present paper is to describe our routine screening procedure and some experiments upon the growth of the tumour under various experimental conditions which provide a background for the critical evaluation of those results.

The earlier history of the Walker tumour has been described by Earle (1935). It was obtained by us from Professor Haddow and has been maintained for several years in these laboratories by serial subcutaneous implantation at eight to twelve days' intervals in albino rats of a heterozygous stock in breeding here. Both stock rats and those upon experiment are housed upon sawdust in galvanized wire mesh cages and are allowed to feed *ad libitum* upon a composite diet in pellet form with tap water available at all times; the diet, which is obtainable from Scottish Agricultural Industries, Limited, has the following percentage composition by weight:

Fine wheat middlings	19.2	White fish meal (60% protein)	4.7
Ground wheat	19.2	Dried skimmed milk	*7.0
Sussex ground oats	19.2	Dried yeast	1.2
Ground barley	9.5	Sodium chloride	0.5
Ground maize	9.5	Cod liver oil	0.5
Meat and bone meal	9.5		

* Increased in later batches to 14%

Our standard procedure for the detection of tumour growth inhibitory activity is similar to that described by Haddow and Robinson (1937) and is essentially as follows:

A rat carrying an actively growing implant, eight to twelve days old, is killed by fracture of the spinal cord and the tumour exposed under aseptic conditions. Fragments cut from the healthy peripheral part and as nearly equal in size (200–300 mg.) as can be judged by the eye are implanted by trochar and cannula, subcutaneously, in the right flanks of a number of rats, each 90 to 120 g. in weight. Each rat receives a single implant and the skin wound is closed with a Michel clip. The rats are then separated into groups of from ten to fifteen animals, matched in respect of sex distribution and mean body weight.

Where it is found necessary to use more than one tumour to provide implants for all the animals in any one experiment, the implants from each tumour are distributed equally

between the several groups. Compounds under test are usually given by intraperitoneal injection, started on the day following implantation and continued daily, Sundays excepted, for the first ten to twelve days of experiment. Occasionally only one dose is given and that within the first few days of the operation. The treated rats are weighed daily and the doses adjusted for body weight changes. The control animals remain untreated, since we have found that the vehicles which we use in making up compounds for injection have no effect upon the growth of the tumour in the quantities employed. On the fourteenth or fifteenth day of experiment, the day of implantation being taken as day 0, all the animals surviving the experiment are weighed and killed and the tumours dissected out, cut in several planes, " blotted " on absorbent wool, and weighed.

Early in the course of our experiments it became apparent that although conditions had been standardized as far as was practicable the tumours which developed in rats implanted with tissue from any one tumour and treated in a manner identical in all other respects varied considerably in size at fourteen or fifteen days. This applied equally to control animals and those which had been dosed with a compound. Examination of the results from a large number of experiments showed that the distribution of tumour weights within groups of rats treated alike was rather unusual. For control animals it was bimodal with one peak between 0 and 2 g. and the other at about 26 g. (Fig. 1).

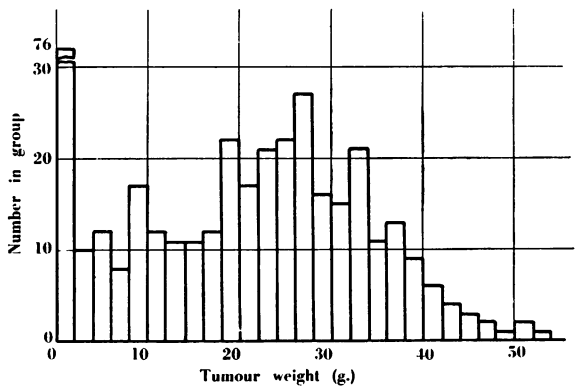

FIG. 1.—Distribution of tumour weights in control animals.

The proportion of rats contributing to the lower peak was about one-third of the total, including animals in which the tumour did not " take," while the distribution of the remaining weights was almost normal. This finding was consistent with the supposition that about one-third of our stock were highly resistant to the growth of the tumour while the remainder were considerably more susceptible, giving, when untreated, tumours weighing up to 50 g. or more.

With these considerations in mind several different methods were examined in order to define the most sensitive means of assessing the statistical significance of the effect of any treatment. The most sensitive method found was to select from each group of treated or control animals the upper half of the distribution of tumour weights (i.e., the n heaviest tumours out of any group of $2n$), to determine the mean, M_{5c}, of each selection, and to assess the statistical significance of the difference in the normal way, assuming that the weights chosen are complete samples. The method is equivalent to selecting that half of all the rats which are most susceptible to tumour growth.

The percentage inhibition of tumour growth, I, we define by the formula:

$$I = \frac{(M_{50} \text{ controls} - M_{50} \text{ treated})}{(M_{50} \text{ controls})} \times 100$$

where M_{50} is the mean referred to above.

The choice of method for assessing the significance of the effect of a treatment is governed by the nature of the distribution of the tumour weights. If this changed, as it might well do if, for example, rats of a different stock were used, then the method recommended might no longer be the most sensitive. The growth of the Walker tumour in rats from our colony has of late been more uniform than in our early experiments and the proportion of highly resistant animals seems to be decreasing. It would be rash to assume that this desirable tendency is permanent, but in several experiments we should be justified in utilizing the whole of the tumour weights in both control and treated groups in calculating the magnitude of the effect and its significance.

Sources of variation in tumour growth rate

The variation encountered in the weights of tumours from identically treated animals under the conditions of our test might arise from (*a*) variation in the rats and/or (*b*) factors dependent upon the technique, such, for example, as differences in the size or condition of the implants. In an experiment designed to separate and estimate the contribution from each of these sources, portions of tissues from two tumours, A and B, were implanted subcutaneously in the right and left flanks respectively of each of 20 rats (10 males and 10 females). The weights of the two tumours in each of the animals fourteen days later are shown in Table I. A marked tendency for both implants in any one rat to grow relatively poorly or relatively well is apparent. The analysis of the variations as given by Dr. O. L. Davies is set out in Table II.

TABLE I

TUMOUR WEIGHTS AT 14 DAYS FROM IMPLANTS OF TUMOURS A AND B IN RIGHT AND LEFT FLANKS, RESPECTIVELY, OF 20 RATS

	Tumour weights in grammes																				Total
Right flank (tumour A) ..	19	18	16	16	14	13	12.5	12	11	11	10	10	10	10	4	4	4	3	0.7	0.3	198.5
Left flank (tumour B) ..	16	16	12	12	16	14	14	8.5	16.5	17	16	16	13	8	3	3	3	4	1.1	2	211.1

TABLE II

ANALYSIS OF VARIATION OF FIGURES SHOWN IN TABLE I

Source of variation	Sum of squares	Degrees of freedom	Variance
Original tumours	3.95	1	3.95
Rats	1,131.40	19	59.55
Remainder	107.44	19	5.65
Total:	1,242.79	39	

The difference in the growth of the implants from tumours A and B considered as a whole is not significant. The remainder variation, which includes that dependent upon the technique, is small compared with the variation between rats. The analysed variances are:

Remainder	5.65
Due to rats (59.55—5.65)/2 ..	27.00

It is concluded that there would be no appreciable gain in precision if more than one tumour were implanted into each rat.

The term " variation between rats " as used above covers not only genetically determined differences in resistance or susceptibility to the growth of the tumour but all differences, e.g., of initial age and body weight, of body growth rate, and of nutritional status, such as might conceivably affect the rate of growth of the implants. Experiments to determine the influence of such variables have so far yielded the results described below.

TABLE III

EFFECT OF INITIAL WEIGHT OF RATS UPON WEIGHT OF WALKER TUMOUR IMPLANTS AT 14 DAYS (CONVENTIONS AS IN TEXT)

	Group I			Group II			Group III		
	Initial wt. of rat (g.)		Tumour weight (g.)	Initial wt. of rat (g.)		Tumour weight (g.)	Initial wt. of rat (g.)		Tumour weight (g.)
	♂	♀		♂	♀		♂	♀	
		50	36.0		111	33.0		185	43.0
	57		28.0	108		32.0	258		36.5
	53		27.0	99		32.0	228		34.0
	53		23.0		109	30.0	257		30.0
	47		22.0	102		27.5		177	29.0
	46		21.0		98	27.5		208	29.0
	53		21.0	97		26.5	272		24.0
		47	21.0		94	23.75	264		21.0
		50	20.5	103		22.5	281		18.0
	52		20.0	110		20.5	284		17.5
		48	20.0	97		17.5		208	15.5
	58		16.5	99		13.0	344		12.5
		50	16.0		95	12.75	241		11.5
		47	15.5		91	12.5	314		10.0
	51		8.75		107	12.0		236	2.0
	45		5.0		96	8.5		204	1.0
		49	2.0		99	1.5		220	0.5
	56		0.0	95		1.0	269		0.0
	49		0.0	97		0.0	200		0.0
		43	0.0	99		0.0		224	0.0
		48	0.0	102		0.0		187	0.0
		48	0.0		93	0.0		206	0.0
		49	0.0		97	0.0		197	0.0
		49	0.0		99	0.0		217	0.0
Means	49.9			99.9			236.7		
M_{100}			13.47			14.75			13.96
M_{50}			23.0			25.48			25.83
M_{50} (♂ s)			23.6			26.83			27.25
M_{50} (♀ s)			21.5			23.25			19.9

Influence of initial age and body weight of rats

An experiment was carried out in which the Walker tumour was implanted into rats of three groups differing widely in body weight. Each group contained 24 animals, 12 males and 12 females. The rats in the first group each weighed between 43 and 58 g. (mean 49.9 g.), those in the second group between 91 and 110 g. (mean 99.9 g.), and in the third between 177 and 344 g. (mean 236.7 g.). Four tumours were used to provide the implants and the same number of rats in each group received a single implant from each of these. The rats were killed and the tumours weighed fourteen days later. The tumour weights, arranged in each group in descending order of magnitude, and the corresponding weights of the rats on the day of implantation are shown in Table III.

The mean tumour weights, M_{100}, in the three groups were 13.47, 14.75, and 13.96 g. respectively, and do not differ significantly from one another in spite of the enormous differences between the mean body weights of the rats. The same applies to the mean weights of the upper 50 per cent of tumours, M_{50} in the three groups being 23.0, 25.48, and 25.83 g. respectively. It is concluded that no significant part of the extreme variation encountered in the tumour weights can be attributed to differences in the initial rat weights.

It will be noted that in each group tumour growth appeared somewhat more vigorous in the males than in the females. This may be correlated with the greater rate of growth of male rats as compared with females.

The foregoing considerations suggest very strongly that the variations in tumour growth rate are dependent largely upon genetically determined differences in susceptibility to tumour growth. We have considered attempting to breed by selection a strain of rat of enhanced susceptibility with a view to obtaining more uniform tumour growth, but so far have not done so.

The influence of nutritional status

An interesting early study of the influence of nutritional conditions upon body growth and tumour growth in tumour-bearing animals is that of Moreschi (1909). He transplanted " sarcoma 7 " into mice weighing about 11 g. and then separated the animals into four groups. In the first three each mouse was given daily 1, 1.5, and 2 g. of food respectively, while those in the fourth group were allowed to feed freely. The tumours grew in proportion to the amount of food the mice received. The gross weight gain of the animals was also directly proportional to this. On the other hand the net mouse weight (gross weight − tumour weight) fell in each group, the decrease being small but definite in the animals feeding *ad libitum* and more marked in the undernourished groups. The increase in gross weight of the well-nourished mice was entirely due to the more rapid growth of the tumours in this group. It was noted that the undernourished mice often died at a later juncture than those feeding freely. A similar experiment with mice weighing over 20 g. initially yielded essentially the same result. Experiments of a similar kind with various tumours in rats or mice have been reported by Haaland (1907), Jensen (1909), Rous (1911, 1914), Sweet, Corson-White, and Saxon (1913), and show that with many tumours at least undernourishment of the host leads to reduction in the rate of tumour growth.

The effect of hypophysectomy as well as of underfeeding upon the growth of the Walker tumour has been studied by McEuen and Thomson (1933). In their experi-

ments a reduction in tumour growth rate was produced by hypophysectomy comparable with that obtained by partial starvation of the tumour-bearing rats. It is clear, however, from the work of Elson and Haddow (1947) that a reduction in body growth rate in animals carrying this tumour does not invariably lead to a reduction in tumour growth rate. They implanted the tumour into rats maintained on diets containing 20, 10, and 5 per cent of protein respectively and followed body and tumour growth for periods up to thirteen days. The mean net body weight of the rats receiving the 20 per cent protein diet increased during this period while that of the rats on the two other diets fell, but no significant difference was observed in the rate of growth of the tumour in the three groups. Some observations of Bischoff and Maxwell (1931) and of Bischoff, Maxwell, and Ullman (1931) are of interest in this connexion. They applied *x*-rays to the pituitaries of rats bearing sarcoma 10 or the Hyde rat carcinoma, with the result that, if the irradiation was sufficient to inhibit the growth of the animals, tumour growth was inhibited also. On the other hand they observed no inhibition of tumour growth when body growth was checked by treating the tumour-bearing animals with various toxic substances or by partially starving them.

We have carried out experiments upon the influence of underfeeding upon the growth of the Walker tumour in which the conditions approximate more closely to those of our standard test than any described in the literature.

In a typical experiment the tumour was implanted into rats in four groups each comprising six males and six females. The mean body weight at implantation in each of the four groups was approximately the same and lay between 96.5 and 98.2 g. Food was supplied in the form of our standard pellets broken down to granules of such size that they passed through a ten-mesh screen but were retained by a twenty-mesh screen. It had been observed that rats would pick such granules individually from a food pot and consume them at leisure with

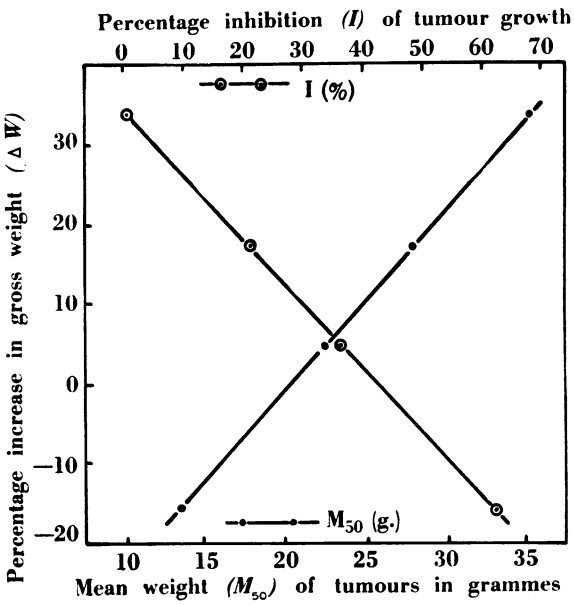

FIG. 2.—Effect of dietary restriction upon tumour growth and gross weight gain in rats bearing the Walker tumour. ●——●, plot of M_{50} against ΔW. ○——○, plot of I against ΔW.

very little wastage. Where, as in this experiment, several animals were kept in a cage with the supply of food restricted, its use in this form ensured that each animal secured its fair share, and as wastage was minimal the daily food intake could be closely controlled. The rats in group I were allowed to feed freely, those in groups II, III, and IV were each allowed 7, 5, and 3 g. of food respectively per day. Restriction of the diet was instituted on the day of implantation of the tumour and continued until the surviving animals were killed and the tumours weighed fourteen days later. Tap water was available at all times.

The results are shown in Table IV. In Fig. 2 the mean percentage increase in gross weight (ΔW) of the surviving animals in each group is plotted against the mean weight (M_{50}) of the heavier 50 per cent of the tumours, and against the percentage

TABLE IV

EFFECT OF UNDERFEEDING UPON GROWTH OF THE WALKER TUMOUR. (CONVENTIONS AS IN TEXT.)
RAT WEIGHTS MARKED * EXCLUDED FROM CALCULATION OF MEANS

	Group I			Group II			Group III			Group IV		
	Rats fed *ad libitum*			Rats fed 7 g. per day			Rats fed 5 g. per day			Rats fed 3 g. per day		
	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)
	Initial	Final		Initial	Final		Initial	Final		Initial	Final	
	92	122	40.0	114	136	32	110	113	25.0	98	86	14.0
	101	147	39.0	102	131	30	78	111	24.0	110	94	13.5
	93	133	37.0	102	120	27	104	108	22.5	105	83	13.0
	109	144	33.5	74	100	27	103	107	22.0	95	92	13.0
	103	125	32.0	91	100	26	98	96	21.0	98	80	10.0
	104	142	29.5	100	120	25	97	98	19.0	89	80	9.0
	90	121	28.0	90	116	24	99	112	19.0	104	84	8.0
	106	125	28.0	104	124	24	100	104	19.0	94	70	4.0
	102	160	26.0	108	114	23	107	92	17.0	99*	Dead	—
	82	116	25.5	104	116	22	97	108	15.0	100*	,,	—
	103	127	25.0	96	93	22	98	94	13.0	82*	,,	—
	84	102	22.0	93	111	22	88*	Dead	—	94*	,,	—
Mean	97.42	130.3		98.17	115.1		99.2	103.9		99.13	83.63	
ΔW		33.7			17.2			4.75			−15.6	
M_{50}			35.2			27.8			22.58			13.4
I			—			21			35.8			61.9

inhibition (I) of tumour growth. It is clear that underfeeding may produce a considerable inhibition of the growth of this tumour, and it appears that over a considerable range M_{50} is proportional to the increase in gross weight of the animals.

In our standard screening experiments all the animals are allowed free access to a diet of fixed composition, but the consumption or assimilation of the food or both undoubtedly varies from group to group under the influence of the compounds given. Any treatment which significantly reduces body growth rate, either by reducing food consumption, impairing the digestive and assimilative functions, or by more obscure and subtle means, is likely to reduce the growth rate of the tumour, although, in view of the observations of Elson and Haddow (1947), it need not necessarily do so. When the administration of a compound is found to lead to inhibition of tumour growth it is desirable to know to what extent that inhibition is merely a manifestation of some such

general " nutritional " or " toxic " effect, and to what extent it is in any sense directed preferentially towards the tumour. From Fig. 2 we can roughly estimate the probable upper limit of the tumour inhibition due to general " toxicity " associated with a given reduction in gross weight gain. It appears that underfeeding at the level required to prevent any increase in mean gross weight inhibits tumour growth by about 40 per cent. This has been several times confirmed. In many of our experiments the dosage of compounds under test has been so adjusted that the mean gross weight of the treated tumour-bearing rats has been held roughly constant throughout. We conclude as a first approximation that any compound which in these circumstances produces inhibition in excess of this figure must act in some way other than by a non-specific " toxic " effect.

Some examples of the various types of response encountered in routine experiments are shown in Table V. The majority of compounds examined produce no greater inhibition of tumour growth than can be accounted for on the basis of non-specific toxicity, as indicated by the effect on gross weight gain. Stilboestrol is of this type, and

TABLE V

THE EFFECT OF SOME COMPOUNDS UPON GROSS WEIGHT GAIN AND THE GROWTH OF THE WALKER TUMOUR IN RATS

Compound	Total dose per 100 g. rat, given over 10–12 days	ΔW		M_{50}		I
		Controls	Treated	Controls	Treated	
Stilboestrol	225 mg. i.p. in oil	31.8	7.5	20.9	18.0	14.0
N-Benzoylethylene-imine	10 mg. i.p. in oil	38.3	13.9	35.3	34.7	1.7
8-Azaguanine	200 mg. i.p. in aqueous suspension	36.0	1.0	29.3	12.9	56.0
Trimethylolmelamine	250 mg. i.p. in aqueous suspension	31.2	14.9	30.3	0.5	98.0
Tris-ethylene-imino-triazine	0.14 mg. i.v. in aqueous solution	28.5	14.3	37.2	2.0	95.0

a result obtained with large doses is shown. A small number of substances has been found which reduce the gross weight gain of tumour-bearing animals without producing any appreciable inhibition of tumour growth. This result is analogous to that obtained by Elson and Haddow (1947) by reducing dietary protein. One such substance is N-benzoylethylene-imine. The compound 8-azaguanine (" guanazolo ") has been reported by Kidder, Dewey, Parks, and Woodside (1949) to retard the development of several mouse tumours. This is one of a number of substances which only inhibit the growth of the Walker tumour to a slightly greater extent than can be attributed to non-specific toxicity. Compounds which are of more particular interest as potential therapeutic agents, however, are those with a clearly specific action upon the tumour. Trimethylolmelamine and 2 : 4 : 6-tris-ethylene-imino-1 : 3 : 5-triazine (T.E.T.) are examples of this type.

SUMMARY

A technique for the screening of compounds for tumour growth inhibitory activity using the Walker carcinoma 256 in rats is described. The influence of various factors upon the growth of this tumour has been studied. The tumours which develop

in fourteen days from subcutaneous implants in albino rats of heterozygous stock vary considerably in size from rat to rat; this variation is mainly due to inherent differences in susceptibility to tumour growth.

Tumour growth is virtually independent of the age and weight of the rats at implantation; it is rather more vigorous in males than in females and is reduced by underfeeding. The inhibition of tumour growth produced by underfeeding is proportional to the reduction in gross weight gain of the tumour-bearing rats. Underfeeding at the level required to prevent any increase in gross weight inhibits tumour growth by about 40 per cent.

Compounds which interfere with body growth may be expected to inhibit tumour growth as a result of their general " toxic " action. This inhibition is unlikely to exceed that which would result from underfeeding at a comparable level. Any inhibition which a compound may produce in excess of that which can be attributed to interference with body growth must be due to factors other than non-specific " toxicity."

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