Table I shows that ingestion of the azo carcinogen led at first to a decline in the serum PPD oxidase levels of the experimental rats. The difference between the mean levels of PPD oxidase activity for experimental and control groups, already pronounced at 21 days, was very marked at 62 days from the start of the feeding experiment. Thereafter, in spite of continued ingestion of the azo dye, serum PPD oxidase levels of the experimental group began to rise and by 154 days there was no significant difference between the mean levels of this enzyme in the experimental and control groups.

When large liver tumours were present in the azo-fed rats, serum PPD oxidase levels remained high (0·4–0·6) so there is no question of permanent suppression or deletion of the enzyme during hepatocarcinogenesis. MILLER and MILLER[3] found that the ability of rats to bind azo dye to their liver proteins began to decline after the rats had been fed for about 2 weeks on a diet containing 3'-MeDMAAB. ARCOS and ARCOS[4] observed that the swelling ability of liver microsomes decreased to a minimum at 4 weeks with the feeding of 3'-MeDMAAB and again increased until normal levels of swelling were attained after 20 to 24 weeks of dye feeding. A diet containing the non-carcinogen 2-methyl-4-dimethylaminoazobenzene failed to influence the swelling of rat liver microsomes after 4 weeks. ROY, MIYA, and CARR[5] found that the total non-protein sulphydryl content of the livers of rats fed 4-dimethylaminoazobenzene reached a minimum value after 12 weeks on the diet. After 20–24 weeks, however, the sulphydryl content had returned to normal levels. In the present experiment we have indications that yet another early effect of the azo carcinogen appears to be overcome as dye-feeding is continued.

Although single intraperitoneal injections of 3'-MeDMAAB as well as of other hepatocarcinogens may produce a temporary increase in serum XD, no significant difference between the mean XD levels of experimental and control groups was found after 21, 62, 120, and 154 days of dye-feeding. It was found however that XD levels of azo-fed rats began to decline within a few weeks after the end of azo dye-feeding. This trend is shown in Table II which also records XD values for control animals. Serum XD levels began to decrease during liver tumour growth and we have noted in another series of rats which had been on a diet of 4-dimethylaminoazobenzene that low serum XD values may be encountered even before liver tumours are palpable. It is of interest that the growth of a transplantable sarcoma Rd/3 in the same rat strain leads to a marked depression in serum XD activity.

Finally, reference is made to the anemic condition of rats fed 3'-MeDMAAB. After 120 days on the diet, hemoglobin levels (as measured by the method of VANDENBELT *et al.*[2] and expressed as an optical density difference at 630 mμ) ranged from 0·229 to 0·254 (4 rats) with a mean value of 0·242. The normal group (5 rats) had values in the range 0·276–0·283 with mean = 0·279. Application of the t test gave $t = 6{\cdot}8$ ($> 0{\cdot}1\%$ level) showing that the difference between the means is highly significant. The azo-fed rat excluded from our analyses had a hemoglobin level of 0·195. Essentially the same results were obtained after 154 days on the diet.

The azo carcinogen has a weak methemoglobinogenic action when given orally to rats (a mean value of 1·7% as compared with 0·2% for the control group at 120 days from the start of dye-feeding) which confirms our previous finding[6] of this condition following single intraperitoneal injections of 3'-MeDMAAB into rats.

It is noteworthy that all the azo-fed rats developed liver tumours which proved to be cholangiocarcinomata. Attempts to transplant several of these tumours met with no success.

I should like to thank Dr. F. N. GHADIALLY for examining histological sections of the tumours, and I am indebted to the University of Sheffield for the James Morrison Fellowship in cancer research.

W. J. P. NEISH

Cancer Research Unit, The University, Sheffield, May 5, 1959.

Zusammenfassung

Werden Ratten mit dem carcinogenen 3'-Methyl-4-dimethylaminoazobenzol gefüttert, so nimmt die Konzentration der p-Phenylendiaminoxidase im Serum anfangs rasch ab, um am Ende der Fütterungsperiode langsam wieder auf den Normalwert anzusteigen.

Eine Verminderung der Xanthindehydrogenase des Serums war nur zu Beginn des Wachstums von Lebertumoren zu beobachten. Mit Azofarbstoffen gefütterte Ratten wurden anämisch.

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Antibodies in the Course of Resorption of the Ehrlich Cancer Heterotransplant in Rats

Mice tumours heterotransplanted to newborn white rats[1,2] or to white rat embryos[3,4] at first grow at a high rate and attain a large size, while in more than two-week old rats they degenerate and are completely resorbed. Resorption of the tumour heterotransplants under these conditions is indicative of lack of acquired tolerance[4], although tolerance to skin homotransplants can be elaborated in less than 2-week old rats[5,6]. The mechanism of transplant resorption in 2-week old rats is still obscure.

In the present study, we have followed up the dynamics of antibodies against tumour tissue in sera of rats with resorbing Ehrlich cancer transplant. For this purpose, the agglutination reaction of tumour cells was tested as well as cytotoxic action of sera upon tumour cells in a test tube and *in vivo-in vitro* neutralization.

Methods. The Ehrlich ascitis adenocarcinoma was subcultured to non-inbred strains of white mice. The ascitis fluid was procured from the mice on the $7-12^{th}$ day after inoculation. The cells were thrice washed with Ringer and 0·25 ml of the suspension of tumour cells containing 160 million cells per 1 ml were injected under the skin of the back of 1–2-day old white rats.

A total of 112 young rats of 12 litters have been inoculated, while 46 normal young rats of 5 litters were used as

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Table I

Agglutination of tumour cells by sera of normal rats and of 1–2-day old rats inoculated with Ehrlich tumour

Age of rats	Time after inoculation	Stage of tumour growth	No. of rats	Agglutination titer
1 week	7 days	Intense growth . . .	13	0
2 weeks	14 days	Cessation of growth .	14	0
3 weeks	21 days	Beginning of resorption	7	1: 80
4 weeks	26-27 days	Resorption of half of tumour	11	1:320
4-5 weeks	25-31 days	Complete resorption of tumour	11	1:320
4-5 weeks	28-35 days	1 week after tumour resorption	9	1:160 1:640
7 weeks	44-51 days	4 weeks after tumour resorption	10	1: 80 1:320
1 week	–	Normal rats	13	0
2 weeks	–	Normal rats	18	0
3 weeks	–	Normal rats	8	0
4 weeks and adult	–	Normal rats	10	0

control. The rats were sacrified 1, 2, and 3 weeks after inoculation, upon a twofold decrease in size of the tumour upon its complete resorption and 1 and 4 weeks after its complete disappearance.

For retransplantation to mice, the tumour growing on young rats was fragmented with scissors, suspended in physiological saline and injected intra peritoneum to 5 mice, 0·2 g tumour tissue to each.

The agglutination of the tumour cells was assayed by the slightly modified HORN[7] method. To a series of 0·2 or 0·4 ml twice diluted serum 0·2 or 0·4 ml suspension of washed tumour cells were added, containing 1 million cells per ml, the mixture was agitated and incubated for a 1 h at room temperature and 18–24 h at + 4°. Agglutination was determined *ad oculo*: +++ was estimated as distinct agglutination at a transparent background. In order to assay the cytotoxic effect *in vitro*[8,9] to 50 million washed Ehrlich tumour cells were added 0·5 ml of the serum to be tested preliminary kept for 30 min at 56° and 0·5 ml of fresh guinea pig serum (complement) taken on the preceding day. The mixture was agitated and incubated at 37° C. 1, 2, and 4 h thereafter samples of the incubated mixture were stained with eosin diluted 1:1000 in a Thyrode solution, 4 ml eosin being added to 0·05 ml suspension of tumour cells. The percentage of stained cells was then estimated under the microscope. To check *in vivo* the *in vitro* cytotoxic 'neutralizing' effect of the sera, the above mixture of cells, serum and complement incubated for 4 h was administered intraperitoneally to 5 mice, 0·2 ml to each.

Results. On the 5–6th day after inoculation, a tumour was palpated in rats 1 × 2 cm in size. It attained the maximal size, viz. 4 × 2 × 2 cm 11–13 days after inoculation, decreasing in size 1–2 days thereafter and completely disappearing 16–31 days after inoculation.

The maximal increase in size and growth stoppage were approximately simultaneous in all rats of every litter.

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Table II

The cytotoxic effect upon tumour cells of serum of normal rats and of those inoculated with Ehrlich tumour

Age of rats	Stage of tumour growth	% of eosin stained cells 1 h after incubation	% of eosin stained cells 2 h after incubation
1 week	Intense growth	3%	4%
2 weeks	Cessation of growth. . .	81%	90%
3 weeks	Beginning of resorption .	95%	100%
4 weeks	Resorption of one half of tumour	98%	100%
4-5 weeks	Resorption of the whole tumour	90%	92%
4-5 weeks	1 week after resorption .	98%	100%
7 weeks	4 weeks after resorption	90%	90%
4 weeks	Resorption of one half tumour (without complement)	20%	18%
1-3 weeks	Normal rats	3–4%	8–12%
4 weeks	Normal rats	10%	10%
Adults	Normal rats	10%	10%

Tumour resorption proceeded at a different rate in rats of the same litter.

The results obtained in the agglutination reaction of tumour cells by rat sera are summarized in Table I.

The agglutinins appear in the rat serum on the 3rd week after inoculation at a titer of 1:80; 2 weeks after inoculation these antibodies are not yet apparent. In the half tumour resorption group, and in that of complete tumour resorption, the agglutinin titer is 1:320, in that of 1 week after complete tumour resorption 1:160 and 1:640, 1 month after complete resorption 1:80 and 1:320. In most of the sera, the zone phenomenon is apparent: no agglutination by the whole serum and in dilution 1:5 is to be noted.

Sera of young rats with resorbing tumour did not agglutinate mice erythrocytes.

The results of *in vitro* determination of the cytotoxic effect are summarized in Table II. 1–2 h incubation with the serum of rats 2–3 weeks after inoculation and of those with resorbing tumour called forth a distinct cytotoxic effect and increased the percentage of eosin staining cells to 81–100%. Control sera, viz. those of non-inoculated rats of the same age, as well as sera of adult rats and heated experimental sera without complement, did not exhibit any such effect. After a 4-h exposure, no difference between the control and experimental sera could be noted as part of the controls caused an increase in the percentage of stained cells up to 70–90%.

The survival time of mice inoculated with tumour cells incubated with the complement and serum of rats with completely resorbing tumour (a total of 35 mice) averaged 22·7 ± 1·61 days; whereas the survival time of 17 mice inoculated with tumour cells mixed with serum of control rats and complement was 12·7 ± 0·27 days. This difference is statistically significant.

Re-transplantation to mice showed that even the remnants of resorbed tumour can be effectively transplanted to mice.

It will appear from the above evidence that even 2-week old rats can produce antibodies. Sufficient amounts of *in vitro* detectable antibodies appear during the period when no symptoms of decrease in size of the tumours are yet apparent, viz. on the 2nd week of postnatal life. The

agglutinins appear in the serum at a later date, viz. 3 weeks after inoculation when resorption of the tumour is in full sway. Cytotoxins and agglutinins are possibly different antibodies.

The question remains open as to the role of these antibodies in the resorption of the heterotransplant. Retransplantation of the tumour cells from rats to mice shows that at least part of the tumour cells remain viable in the organism which contains cytotoxins.

Resorption of the mice tumour and appearance of cytotoxins ensues in 2-week old rats while tolerance to homotransplantation of skin is still demonstrable (BILLINGHAM and BRENT[6], WOODRUFF[5]).

The capacity of 2- and 3-week old rats to produce antibodies against heterologous tumours throws doubt upon the widely accepted opinion of 'immunological immaturity' of rats of this age.

It is interesting to compare our data and work of HOLLIDAY[10], who has vaccinated 10–11-day old rats with *S. Pullorum* and observed antibody production.

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The L. A. Tarasevich State Control Institute of Vaccines and Sera, Moscow, January 27, 1959.

Résumé

Chez les rats jeunes auxquels on a inoculé le cancer ascitique d'Ehrlich 1–2 jours après la naissance, la tumeur atteint une grande dimension et disparaît au bout de 14–31 jours. Chez des rats de 14 jours, le sérum a une action cytotoxique sur les cellules du cancer d'Ehrlich et agglutine celles-ci à partir du 21e jour.

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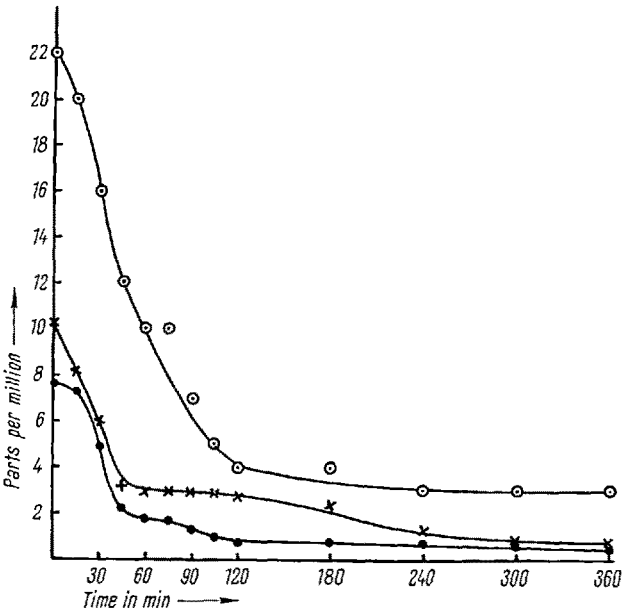
Rapid Removal of Phosphorus from Sewage by Activated Sludge

In a study of the growth of plants in the activated sludge tank by setting up what may be termed 'hanging gardens', it was interesting to observe the behaviour of the rice plant which showed an extraordinary vegetative growth, attaining an unusual height and putting forth more than fifty tillers but showing poor formation of grain[1]. The rice plant appeared to suffer from deficiency of certain nutrients, notably phosphorus, while it had an excessive or abundant supply of nitrogen. Under the conditions of the experiment, the plants floated on the liquid in the last aeration chamber of the activated sludge tank (the plants being in a suitable box equipped to float, containing a bed of resistant cellulosic material such as *coir* fibre and straw bits) and derived their nutrition from the liquid. The sewage entering the purification tank contained a considerable amount of water-soluble phosphorus, but, as it arrived at the last aeration chamber of the tank after about 4 h of detention with activated sludge, the soluble phosphorus was apparently little or inadequate. It was therefore of interest to study the rate of removal of phosphorus, particularly water-soluble phosphorus, during the purification process. As there is practically no information on these points in the literature on the subject[2], the evidence we have collected is briefly described in this communication.



Rate of removal of phosphorus from sewage by activated sludge •——• water soluble phosphorus (P); ×——× total phosphorus (P); ⊙——⊙ 3 min permanganate value.

Experiments were carried out by adding varying amounts of activated sludge to samples of detritus-free raw sewage (containing generally 10 to 14 p.p.m. of total phosphorus and 6 to 9 p.p.m. of water-soluble phosphorus as determined by the method described by FISKE and SUBBAROW[3] and modified by KING[4]; for the determination of water-soluble phosphorus the samples were filtered through filter paper No. 44) and by blowing air through these mixtures (in suitable conical flasks) for varying periods, generally up to 6 h, and by examining the supernatant liquids for their water-soluble and total phosphorus contents, as also for the degree of purification, by customary methods. At the same time, the effect of aeration of samples of sewage only (without the sludge) on their phosphorus contents was also studied.

In one series of experiments, the sewage samples were aerated with 25% sludge for varying periods, from 15 min to 6 h, and the supernatant liquids (collected after stopping the aeration at stated intervals and allowing the contents of the flasks to settle down) were analysed for their phosphorus contents and for the extent of purification as indicated by the 3-min oxygen absorption test (permanganate value) and turbidity. These observations are given in the Figure. In a similar series of experiments, the concentration of sludge was varied while the period of aeration was the same (Table).

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