prevented hemolysis, but also prevented the demonstration of rods and granules when blood so treated was examined in either wet or dried films.

The identity of the cells containing rods and granules with reticulocytes could be demonstrated by adding a small amount of brilliant cresyl blue or new methylene blue[2] to the oxalate solution used in preparing wet films. When this was done, the rods and granules were incorporated into stainable reticulum. Even small amounts of these basic dyes stained the reticulocytes very rapidly and only an occasional cell could actually be observed in the process of staining. From such observations it appeared that some of the granules seen in unstained preparation corresponded to the metachromatic granules seen in the midst of the reticulum when stained with basic dyes.[3] The reticulum, however, appeared frequently independently of the preexisting granules, although these granules may be incorporated into the meshes of the reticulum.

Preliminary experiments demonstrated essentially similar rods and granules in human reticulocytes.

[2] Brecher, G., unpublished data.

[3] Cesaris-Demel, A., *Folia hemat.*, 1907, **4**, Suppl. 1, p. 1.

16626

Influence of Interrupted Carcinogenic Treatment on Tumor Formation.*

H. P. RUSCH AND B. E. KLINE.

From the McArdle Memorial Laboratory, University of Wisconsin Medical School, Madison, Wisconsin.

There is considerable evidence that the formation of cancer occurs as a series of biological changes.[1-5] This concept is supported by several types of experimental data but of special consequence is the evidence of a latent period under certain conditions of carcinogenesis. When a carcinogen is applied to the skin of mice for a time short of that necessary to induce tumors, the subsequent resumption of the carcinogenic stimulus will quickly evoke the formation of tumors even though 2 to 3 months intervene between the 2 periods of treatment.[6] Actually a noncarcinogenic agent, such as heat, a wound or croton oil, may substitute for the carcinogen in the second period.[7-8] Such observations indicate that the initial application of the carcinogen produces alterations in the cells that persist for a considerable period. Whether the initial cellular changes revert gradually or persist indefinitely is not known. In order to test this point, rest periods from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. It was thus possible to obtain some information on the reversibility of the process.

Procedure. This investigation may be divided into two parts: in one, methylcholanthrene was employed to initiate the process

* This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council and by a grant from the Jonathan Bowman Fund for Cancer Research.

[1] Berenblum, I., and Shubik, P., *Brit. J. Cancer*, 1947, **1**, 383.

[2] Friedewald, W. F., and Rous, P., *J. Exp. Med.*, 1944, **80**, 101.

[3] Rusch, H. P., *Physiol. Rev.*, 1944, **24**, 177.

[4] Kline, B. E., and Rusch, H. P., *Cancer Res.*, 1944, **4**, 762.

[5] Rusch, H. P., and Kline, B. E., *Arch. Path.*, 1946, **42**, 445.

[6] Lavik, P. S., Moore, P. R., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, 1942, **2**, 189.

[7] Des Ligneris, M. J. A., *Am. J. Cancer*, 1940, **40**, 1.

[8] Berenblum, I., *Arch. Path.*, 1944, **38**, 233.

and, after varying periods of rest, croton oil, a non-carcinogenic agent, was used to evoke the neoplasms; in the other ultraviolet irradiation was used for both periods of treatment.

Methylcholanthrene - croton oil. Young adult strain C mice of both sexes were used. They were kept on shavings in groups of 20-24 in metal box cages with a stock grain ration and water available at all times. A solution of 0.2% methylcholanthrene in benzyl alcohol was painted on the backs of all mice twice weekly for 19 weeks. At this time approximately 10% of the animals had papillomas. The mice with papillomas were discarded and those free of visible tumors were divided into 6 groups of 42. These were then treated as follows: One group received no further treatment, another had the hydrocarbon applications continued for 13 weeks, and in a third group a 0.5% solution of croton oil in benzyl alcohol was applied to the precarcinogenic area, 3 times a week for thirteen weeks. The remaining 3 groups were also painted with croton oil in the same manner as the latter group except that rest periods of 4, 9, and 13 weeks elapsed between the cessation of methylcholanthrene application and the start of the treatment with croton oil. The incidence of tumors was checked every 2 weeks and they were classified as papillomas and carcinomas.

Ultraviolet Irradiation. Young adult albino strain C mice of both sexes were used. They were kept on shavings in groups of 24 in ordinary metal box cages with a stock grain ration and water available at all times except during irradiation. At the time for raying, they were transferred to a special cage 25.5 cm square by 3 cm deep, constructed of wire mesh, and divided into 24 individual compartments to prevent the mice huddling together and to minimize movements.[9] Except for rest periods, all mice were irradiated 30 minutes a day, 6 days a week with the light of a medium pressure mercury vapor lamp. The daily amount of irradiation was 3.6 x 10^7 ergs/cm^2 at an intensity of 2 x 10^4 ergs/cm^2

[9] Rusch, H. P., Kline, B. E., and Baumann, C. A., *Arch. Path.*, 1941, **31**, 135

TABLE I. Effect of Rest Periods on Incidence of Carcinomas Following Treatment with Methylcholanthrene and Croton Oil.
(A solution of 0.2% methylcholanthrene (Mc) was applied to backs of all these mice for a period of 19 weeks prior to treatment indicated below).

Group	Subsequent	Tumor incidence—months after start of first application of methylcholanthrene															
		6			7			8			9			10			
		N	T	%	N	T	%	N	T	%	N	T	%	N	T	%
1	Mc continued 3 mo.	41	1	2	38	4	10	25	17	40	12	30	71	1	41	98	
2	Croton oil 3 mo.	40	2	5	34	7	17	21	18	43	11	28	67	2	35	85	
3	Rest 1 mo. then Croton oil 3 mo.	42	0	0	40	2	5	32	8	19	16	21	50	8	28	67	
4	Rest 2 mo. then Croton oil 3 mo.	42	0	0	40	2	5	35	7	17	21	19	45	12	27	65	
5	Rest 3 mo. then Croton oil 3 mo.	42	0	0	38	4	10	30	8	20	23	14	33	18	16	38*	
6	No treatment	42	0	0	41	1	2	34	5	12	27	9	21	21	14	33	

N = Free of malignant neoplasms. T = Malignant neoplasm.
* At 11 months there were:

	N	T	%
	15	18	43

The per cent of tumors is calculated from the number of animals alive at the time the first tumor is noted.

TABLE II.
Effect of Rest Periods on Tumor Incidence Following Treatment with Ultraviolet Irradiation.

Group	Procedure (Time in months)	Tumor incidence - months after start of raying																			
		4			5			6			7			8			9			10		
		N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%
A	Ray 5	22	2	8	18	5	21	10	12	50	4	18	75	0	21	88	—	—	—	—	—	—
B	" 3, Rest 1, Ray 2	23	0	0	22	1	4	13	9	39	3	18	78	2	19	83	—	—	—	—	—	—
C	" 3, " 2, " 2	21	1	5	17	2	9	14	5	23	6	10	45	4	11	50	3	12	55	5	13	54
D	" 3, " 3, " 2	24	0	0	23	1	4	20	1	4	17	3	13	12	7	29	6	12	50	—	—	—
E	" 3, " 1, " 1																					
	" 1, " 1	24	0	0	19	4	17	17	6	26	11	12	52	2	19	83	—	—	—	—	—	—
F	" 3, " 1, " 1	23	1	4	19	4	17	13	10	42	8	15	63	4	19	79	—	—	—	—	—	—
G	" 1, " 1, " 2	23	1	4	23	1	4	19	1	4	19	1	4	18	2	8	11	5	27	—	—	—
H	" 1, " 1	24	0	0	23	0	0	23	0	0	23	0	0	22	0	0	19	0	0	—	—	—
I	" 1	24	0	0	24	0	0	23	0	0	22	0	0	22	0	0	20	0	0	—	—	—
J	" 2	23	0	0	21	1	5	20	1	5	20	1	5	18	2	9	15	2	9	—	—	—
K	" 3	24	0	0	23	1	4	20	2	8	20	2	8	18	3	13	12	5	21	—	—	—
L	" 4	24	0	0	17	6	26	13	10	44	8	13	57	5	15	65	—	—	—	—	—	—

Designations are the same as in Table I.

/sec. The mice were examined for tumors every 2 weeks and were weighed at monthly intervals. Details of procedure for each group are given in Table II.

Results. Methylcholanthrene-Croton Oil. The rates of tumor formation in the various groups of mice are listed in Table I. At the end of 19 weeks of treatment with methylcholanthrene about 10% of the mice had developed papillomas. At this time, the applications of hydrocarbon were stopped and all the animals with visible tumors were discarded. The remaining mice were divided into 6 groups of 42 each and were treated according to the method indicated in Table I.

The formation of papillomas continued at a rapid rate in all groups, and one month after the initial methylcholanthrene had been discontinued, less than half of the mice in all groups were free of benign tumors. The rate of formation of the malignant neoplasms was slower and varied considerably among the groups. In the mice that received no further treatment, the rate was steadily upward and 33% of the animals had carcinomas at the end of 10 months (Group 6). The rate of development of malignant tumors was very rapid in the mice of the control group in which the hydrocarbon was continued for another 3 months (Group 1) and in the mice that were painted with croton oil immediately after the initial period (Group 2). The final incidence of tumors after 10 months in these latter 2 groups was 98 and 85% respectively. In confirmation of the earlier observation of Berenblum[8] croton oil was almost as effective as methylcholanthrene in eliciting tumors in a precarcinogenic area. When periods of one or 2 months were allowed to elapse before the application of croton oil, the development of carcinomas was delayed slightly (Groups 3 and 4), but the rate of tumor formation paralleled that in Groups 1 and 2 following the treatment with the irritant. The final tumor incidence in groups 3 and 4 at 10 months was 67 and 65% but the slope of the curve indicated an eventual incidence close to that in Groups 1 and 2. When three months elapsed between the initial treatment and the start of croton oil, the rate of formation of

neoplasms was slower and the eventual number of tumors obtained was less than in the other groups; the incidence at the end of 11 months being 43%.

Ultraviolet Irradiation. The rate at which tumors appeared in the various groups of mice irradiated with ultraviolet light is given in detail in Table II. The animals remained in good health throughout the experiment and the tumors formed were identical to those previously reported.[10] Mice rayed continuously for 5 months developed tumors rapidly and there was an incidence of 88% after 8 months (Group A). In the next 3 groups the 5 months of irradiation was divided into two periods with an initial irradiation of 3 months separated from the last 2 months by rest periods of one, 2 or 3 months. A rest of one month had little effect on tumor formation, and the results were essentially the same as for the control group (Group B). However, when the rest period was prolonged to 2 or 3 months, the incidence of neoplasms at 9 months was only 55 and 50% respectively (Groups C and D), but the rate of tumor formation in these groups was similar to that in Groups A and B. The rapid response to the second period of raying is evident from the data.

The effect of various other periods of irradiation or rest were also observed. When the total 5 months' irradiation was divided so that the initial 3 months of raying was followed by one of rest, one of ray, another of rest and a final one of irradiation (Group E), the results were similar to those obtained when the initial period was followed by one of rest and 2 of irradiation (Group B). Essentially no difference was seen when the final period of irradiation was only of one month duration instead of 2 (Group F). When 3 months of irradiation were divided so that the initial period of one month was separated from the final period by a month of rest, (Group G), the results were similar to those obtained when they received the same total dose over a continuous period (Group K).

Groups H, I and J illustrate that a minimal amount of irradiation was required to induce tumors. Since this fact had been previously known these groups were employed only for controls of the present experiment. One month of irradiation by itself was insufficient to elicit tumors during the period of observation (Group I). The practical minimal time under the condition of this experiment was 2 months of continuous irradiation (Group J) and the same total dosage divided into 2 equal periods but separated by a month of rest was ineffective (Group H). Mice rayed for 3 months had a tumor incidence of 21% at the end of 9 months (Group K), and 4 months of continuous irradiation (Group L) was almost as effective as 5 months. In retrospect, it appears that the lower incidence of tumors obtained with only 2 months of irradiation would have been a more satisfactory initial dose for testing the effect of rest periods than the longer period used in this experiment. The longer initial period was selected on the basis of previous work that indicated a two month period to be a marginal one.

Discussion. The present investigation strengthens our previous suggestions that the process of tumor formation can be divided into 3 periods.[4,5] The initial phase is a conversion of a few normal cells into a few neoplastic cells and has been called the Period of Induction[4,5] or the Initiating Process.[1,2] The next stage is the multiplication of such cells into growing visible tumors, and it is the division of this period into two separate stages that we wish to emphasize. The first of these periods is the Critical Period.[5] This starts when the genesis of the neoplastic cell has been completed and is that stage in which cellular proliferation is delicately balanced. During this phase there are so few neoplastic cells present that they are more or less lost among the normal cells, and since their mass has not yet attained a size sufficient to receive a direct supply of blood, they must compete with the healthy cells for the nutrients in the tissue spaces. At such times these cells are the most susceptible to the influence of their environment; they may proliferate, lie dormant, or succumb, or all

[10] Grady, H. G., Blum, H. F., and Kirby-Smith, J. S., *J. Nat. Cancer Inst.*, 1943, **3**, 371.

three processes may occur in the same general area at one time. The fate of small masses of neoplastic tissue depends on the balance between their proliferative capacity and the amount of stimulation or of resistance present in the neighboring tissues. The sum of all these factors determines the rate at which the cells proliferate.[11,12] If the proliferation of the neoplastic cells continues until they attain a size sufficient to establish an independent blood supply, they then become less dependent on local environmental conditions and enter the third phase—that of Progression.[5]

The postulation of a critical period appears essential for the explanation of the results presented in this paper. In the first place, a period of reversibility is indicated because of the decreased incidence of tumors following a long interval between the first and final carcinogenic treatments. Such results may be explained on the basis of the number of cells surviving during the quiescent period. If the rest period is prolonged, some of the neoplastic cells die so that the number able to respond to the second treatment with irritant or carcinogen diminishes as the period of rest increases. Actually, if the rest period is very long, the latent tumor cells may disappear entirely or be reduced to such a level that no visible tumors could develop in the normal life of the mouse even though the proper stimulus is eventually applied. Furthermore, the evidence indicates that nonspecific irritants are effective only during an interval that corresponds to the critical period described in the preceding paragraph. The irritant cannot convert normal cells into malignant ones[1] and since it also is without effect on the growth of visible tumors, it can be assumed that the action is exerted during an intermediate period. The conversion of normal cells into malignant ones is brought about solely by the specific carcinogenic agents,[1] an inference that is borne out by the rapidity by which tumors are evoked following the treatment by croton oil. Evidence from other sources also favors this concept.[2,5,11,12]

Indications for a critical period in this investigation are in contrast to those of Berenblum and Shubik[1] who find that the initial changes are irreversible. They used only one application of 9:10 dimethyl-1:2-benzanthracene and reported only the appearance of warts, whereas we used multiple treatments with methylcholanthrene or ultraviolet irradiation and include only the malignant neoplasms in the data of this paper. Since benign tumors proliferate more slowly it is possible that their life span is longer. To test this point the experiment of Berenblum and Shubik should be repeated allowing a greater period of rest before the irritant is applied.

Summary. The influence of interrupted carcinogenic treatment on tumor formation was tested. Rest periods of from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. The investigation was divided into two parts on the basis of the carcinogens employed. In one, ultraviolet irradiation was used for both the first and second periods. In the other, methylcholanthrene was employed to initiate the process and croton oil, a non-carcinogenic substance, was used to evoke the neoplasms. The results with both technics were essentially the same.

A lag in tumor appearance was observed during the rest periods, and the second period of treatment quickly brought forth tumors in the precarcinogenic areas. Essentially no difference in the rate of tumor formation or in the final tumor incidence was observed when the rest period was of one month duration. When the rest period was increased to 3 months, the final tumor incidence was reduced by 34% in the mice irradiated and by 42% in those in the methylcholanthrene-croton oil series. The results indicate that the initial application of the carcinogen produced alterations in the tissues which were maintained for at least one month, thereafter the changes re-

[11] Blum, H. F., *J. Nat. Cancer Inst.*, 1943, **3**, 569.

[12] Blum, H. F., *J. Nat. Cancer Inst.*, 1944, **4**, 559.

verted toward normal. It is suggested that the genesis of the neoplastic cell was completed during the initial treatment, and such cells remained dormant or divided at a decreased rate until further stimulated. The non-specific irritant, croton oil, exerts its effect during the critical period of carcinogenesis.

16627

Antigenic Relationship of *Salmonellae* to Inaba Strains of *Vibrio comma* Isolated in Egypt.

OSCAR FELSENFELD.

From the Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Ill.

The sera of patients infected with *Salmonellae* frequently agglutinate cholera vibrios.[1] Gohar and Makkawi,[2] studying *Vibrio comma* strains from the recent outbreak in Egypt, found that these organisms were agglutinated by *Salmonella enteritidis* sera. It seemed of interest, therefore, to investigate which antigenic factors are common to *Salmonellae* and cholera vibrios.

Three strains of *V. comma* of the Inaba type, isolated from the recent cholera epidemic in Egypt, were used in these experiments.* *Salmonella* "O", "Vi" and "H" sera prepared for routine *Salmonella* grouping (typing) by methods described by Edwards a.o.[3-5] were employed. Procedures recommended by Ranta and Dolman[6] were used for the production of *V. comma* "OH" sera. Agglutination and absorption tests were carried out according to the standard methods employed for *Salmonella* grouping (typing).[4,5]

Live, alcohol-treated ("O") and formolized ("H") antigens prepared from the cholera vibrios were tested against 25 "O", 2 "Vi" and 39 "H" *Salmonella* sera. Agglutination of the vibrios occurred with dilutions of these

TABLE I.
Results of Agglutination Tests Using *Salmonella bredeney* "O" Serum.

Agglutination (after absorption) with "O" antigen from	Serum absorbed with "O" antigen from							
	None	*S. bredeney* I, IV, XXVII, XII	*S. paratyphi* A I, II, XII	*S. pullorum* IX, XII	*S. worthington* I, III, XXIII	*V. comma* "k"	*V. comma* "4"	*V. comma* "8"
S. bredeney I, IV, XXVII, XII	16*	0	6	4	4	12	12	12
S. paratyphi A I, II, XII	8	0	0	4	2	4	2	2
S. pullorum IX, XII	6	0	0	0	6	1	2	2
S. worthington I, III, XXIII	5	0	1	5	0	3	2	2
V. comma "k"	2	0	0	1	1	0	0	0
V. comma "4"	4	0	0	1	1	0	0	0
V. comma "8"	2	0	0	2	1	0	0	0

* All agglutination titers to be multiplied by 100.

1 Felsenfeld, O., and Young, V. M., A.A.A.S. Annual Meeting, 1946.

2 Gohar, M. A., and Makkari, M., *J. Trop. Med. and Hyg.*, 1948, **51**, 95.

* These strains were received through the courtesy of Dr. Harry Seneca of Columbia University.

3 Edwards, P. R., and Bruner, D. W., *Kentucky Agri. Exp. Sta. Bull.*, 1942, **54**.

4 Felsenfeld, O., *Am. J. Clin. Path.*, 1945, **15**, 584.

5 Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1946, **52**, 493.

6 Ranta, L. F., and Dolman, C. E., *Can. J. Publ. Health*, 1943, **34**, 26.