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INHIBITION OF N-2-FLUORENYLDIACETAMIDE INDUCED HEPATIC CARCINOGENESIS IN RATS BY CHLORAMPHENICOL: A DOSE-RELATED PHENOMENON WITH REDUCED PROTEIN BINDING OF CARCINOGEN‡

In 1965 a preliminary study revealed the protective effect of oral chloramphenicol in adult male Wistar rats against hepatic carcinogenesis and cirrhosis induced by feeding 0.05% N-2-fluorenyldiacetamide in a semisynthetic diet of Morris.[1] The chloramphenicol was fed at the level of 2 percent in the diet as the maximal tolerated dose according to the recommendation of Dr. A. C. Bratton, Jr., Director of Pharmacological Research at Parke, Davis, and Company who donated the chloramphenicol for both the preliminary and the present study.

Once the preliminary study had demonstrated the effectiveness of a maximal dose of chloramphenicol, it was decided to determine whether such a large dose was necessary and at what level of dosage significant inhibition could be obtained, using the same model that had been employed in this laboratory for many years in studies of the role of sex and various hormones in hepatic carcinogenesis.[2] Also, to what extent was the carcinogen and/or its metabolites bound to protein in the liver cells?

PROCEDURE

EXPERIMENT I

One hundred male F_1 hybrid rats, each weighing between 150 and 175 grams, offspring of inbred AXC males and commercial Sprague-Dawley females, were selected and divided into four groups of 25 rats each. The animals were fed a semi-synthetic diet of Morris (Table 1) to which was added 0.025 percent N-2-fluorenyldiacetamide¶ and varying concentrations (0-control, 0.1%, 0.5% and 2%) of chloramphenicol. The diet was fed by modified pair-feeding with 5 rats per cage. The amount of diet offered in each cage was controlled by the amount eaten per day by the control group given carcinogen but no chloramphenicol in the diet. This control group ate the least and all the other groups consumed all of the limited diet offered. The animals had free access to water at all times. Each animal was numbered and weighed weekly throughout the experiment.

The carcinogenic diet was fed for three periods of four weeks each with one week between each of the four-week periods when the rats were fed the same semi-synthetic

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‡ Project supported in part by a grant from the American Cancer Society.

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Received for publication 28 April 1970.

TABLE 1. MORRIS DIET

Commercial casein	300 gm.
Skim-milk powder	2,275 "
Ground, hard spring wheat	6,152 "
Brewer's yeast	200 "
Desiccated whole-liver powder (Wilson)	200 "
Sodium chloride	140 "
Ascorbic acid	10 "
Ferric citrate	13 "
Cod-liver oil	100 "
Corn oil	610 "

diet and dose of chloramphenicol but no carcinogen. Following the twelfth week of carcinogen the animals received two weeks of semi-synthetic diet containing the appropriate dose of chloramphenicol but no carcinogen and then all of the rats were placed on Purina Laboratory Chow pellets *ad libidum*.

The animals were kept alive for up to 68 weeks, after which they were killed and examined. All rats were autopsied and tissues were fixed in 10 percent formalin. Blocks of liver and lungs from each animal were embedded in paraffin, sectioned and stained with hematoxylin and eosin. In those animals with lesions in other organs, microscopic sections were similarly prepared for examination. Previous studies[3] had shown that a minimum of 25 weeks was necessary from the time the feeding of carcinogen was begun for the first hepatocellular carcinoma to develop. Consequently, four animals that died prior to 25 weeks were dropped from the experiment and are not included in the results shown in Table 2. All of the animals tabulated lived for 45 weeks or more after the start of the experiment.

EXPERIMENT II

In an attempt to determine to what extent N-2-fluorenyldiacetamide and/or its metabolic products were bound into the protein of the liver cells, a separate short-term experiment was also performed. Forty-nine male Wistar rats and eight females, weighing 200 to 250 grams each, were divided into four groups. Twenty males were fed the basic semi-synthetic diet without carcinogen or chloramphenicol. Three were killed each week for the first four weeks and two each succeeding week through 8 weeks; they served as baseline controls. Seventeen male rats were fed the diet containing carcinogen and were sacrificed at weekly intervals, generally two to three rats at a time for four weeks and three more at eight weeks. The eight female rats were treated similarly, with analysis of the liver protein from two animals per period. The remaining twelve male rats were fed 2 percent chloramphenicol in the carcinogen-containing diet and sacrificed periodically, generally in pairs up to four and a half weeks. Six rats were killed at 8 weeks following the start of the experiment. The amount of protein-bound carcinogen or its metabolic products in the liver was determined by the method detailed below.

At the time of sacrifice of each animal the liver was removed, protein extracted and ultraviolet absorption at 554 mμ was measured as an index of fluorenyl content according to the following procedure: The rats were anesthetized with ether. The abdomen of the rat was opened and the liver perfused with 35 ml. of cold 0.9% sodium chloride by injecting the portal vein after cutting the hepatic artery and inferior vena cava. The

liver was then removed, rinsed in cold 0.9% saline and blotted with absorbent paper, weighed, and 2 gm. aliquots were minced finely with scissors. The minced liver was placed in ten times its volume of saline and ground in a Duall homogenizer. The liver proteins were precipitated from the homogenized liver suspension by the addition of an equal volume of cold 10% trichloracetic acid (TCA). The liver proteins were sedimented by centrifuging in an International refrigerated centrifuge at 2,500 rpm for 15 minutes. The supernatent fluid was discarded and the precipitated proteins were washed again with 10% cold TCA. The TCA-precipitated liver proteins were then washed with cold 95% ethanol-chloroform (3 to 1 by volume), ethanol-diethyl ether (3 to 1 by volume) and diethyl ether. The granular, tissue-protein residue was placed in a preweighed evaporating dish, dried in a vacuum dessicator over calcium sulfate for 24 hours and weighed. Approximately 0.25 grams of powdered liver protein was obtained from each 2 gram aliquot of rat liver by this extraction procedure. Aliquots from this powdered tissue-protein extract were used for determination of fluorenyl content.

Fifty milligrams of dried rat liver protein from rats which had been fed the Morris diet containing N-2-fluorenyldiacetamide were placed in screw-capped test tubes containing 5 ml. of a mixture of two volumes of 95% ethanol and three volumes of concentrated hydrochloric acid. The acid-alcohol mixture containing the rat liver protein was placed in a 70°C. water bath and allowed to incubate for 1½ hours. A reddish-purple color developed in the solvent from the rat liver protein samples that were taken from the rats which had been fed the semi-synthetic diet that contained N-2-fluorenyldiacetamide. The color developed slowly and reached a peak intensity after approximately 45 minutes of incubation. A spectral analysis was made using a Beckman DU spectrophotometer and the peak absorbancy of the liberated color occurred at 554 millimicrons, which corresponds to the color and absorption of known fluorenyl compounds studied in this laboratory.

RESULTS

EXPERIMENT I

All of the groups of animals lost weight initially but in 2 to 3 weeks they had regained their loss. Thereafter, there was a progressive gain in weight which began to level off at about 40 weeks and after about 55 weeks there was a gradual loss of weight (Figure 1). The growth curves are quite similar among the four groups except terminally when the control group, which had the highest incidence of tumors, began to lose weight quite rapidly and averaged 48 grams per animal less than the most protected group on 2 percent chloramphenicol, 38 grams less than the group on 0.5 percent chloramphenicol and 22 grams less than the group on 0.1 percent chloramphenicol (Table 2).

The main findings at autopsy of the four groups of rats are shown in Table 2.

In the control group receiving carcinogen but no chloramphenicol, 24 animals survived from 52 to 68 weeks. Of these, 18 lived 68 weeks, 16 developed hepatocellular carcinomas, and 1, hyperplastic nodules. In the six animals that expired prior to 68 weeks, three had tumors and two, hyper-

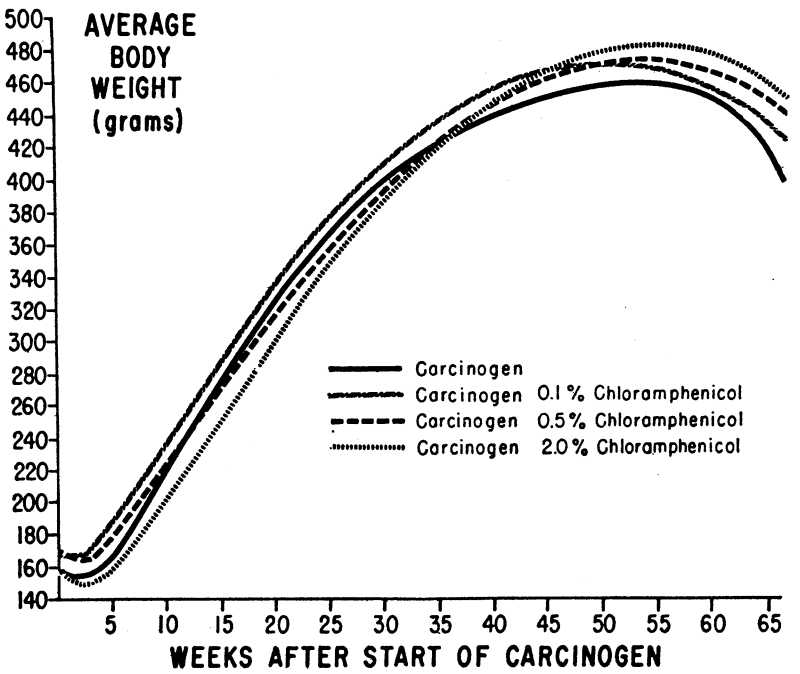

FIG. 1.

plastic nodules. In this group, three animals developed pulmonary metastases from their hepatic carcinomas. All showed cystic changes in the liver, varying from minimal (10 animals) to moderate or extensively involved (14 animals). Five animals had neoplasms other than hepatocellular carcinomas in this group. They consisted of three bronchoalveolar cell tumors of the lung, one squamous cell carcinoma of the ceruminous gland of the ear and one bile duct adenoma. The liver weights varied from 21 to 106 grams and averaged 50.9 grams. Cirrhosis was not present in this or any of the subse-

TABLE 2. RATS FED 0.025% N-2-FLUORENYLDIACETAMIDE FOR 12 OF 14 WEEKS

Chloramphenicol fed 16 weeks	*Animals*	*Final avg. wt. difference*	*Avg. liver wt.*	*Hepatocellular carcinomas*		*Cysts*	*Other tumors*
percent	*number*	*gms.*	*gms.*	*number*	*%*	*number*	*number*
0	24	—	50.9	19	79	24	5
0.1	23	+22	48.6	15	65	22	2
0.5	25	+38	35.3	15	60	19	1
2.0	24	+48	22.1	4	17	4	8

quent groups, but bile duct cysts were present in the liver of all of the animals in this group.

Twenty-three animals survived from 45 to 68 weeks in the group that received carcinogen plus 2% chloramphenicol. Sixteen lived 68 weeks and 12 of these had normal livers, two had small hyperplastic nodules and two developed hepatocellular carcinomas. Of the eight animals that expired prior to 68 weeks, two had hepatocellular carcinomas and one of these had pulmonary metastases. Minimal to moderate focal cystic changes were evident in the four animals with liver tumors. Eight neoplasms other than those in the liver were present: Four of these were squamous cell carcinomas from the ear, one was a large subcutaneous fibroma, two were transitional cell carcinomas from the urinary bladder, and one was a collision tumor consisting of both parenchymal adenocarcinoma of the kidney and transitional cell carcinoma of the renal pelvis. The livers varied from ten to thirty-seven grams in this group and averaged 22.1 grams.

In the group receiving 0.1% chloramphenicol in the carcinogen-containing diet, 23 animals lived from 50-68 weeks. Thirteen of 20 rats that were sacrificed at 68 weeks and two of three animals expiring prior to 68 weeks had hepatocellular carcinomas. Two had developed pulmonary metastases from their tumors. In two animals, hyperplastic hepatic nodules were present. Hepatic cystic changes were present in all the animals with liver tumors and in seven without liver tumors. In seven the cystic changes were considered minimal, and in 15, moderate to marked. Two additional tumors, one bronchoalveolar cell tumor of the lung and one subcutaneous fibroma, were present. Liver weights ranged from 30 to 105 grams, and averaged 48.6 grams.

In the remaining group which received 0.5% chloramphenicol with the carcinogen in the diet, 25 animals survived 46 to 68 weeks. Eleven of 18 sacrificed at 68 weeks had hepatocellular carcinomas, while four of seven that had expired previously had such tumors. Two animals had pulmonary metastases. Nine animals showed minimal cystic changes in the livers, and in ten the cystic changes were considered moderate to marked. No hepatic cystic changes were present in three of the rats with hepatocellular carcinomas. Only one additional tumor, a papillary transitional cell carcinoma of the urinary bladder was present in this group. Hepatic weights varied from 16 to 76 grams and averaged 35.3 grams.

The protective effect of chloramphenicol is represented graphically in Figure 2.

Statistical analysis of these results using a modified X^2 test[8] reveals highly significant influence of dosage on chloramphenicol inhibition with a p value of $<<0.001$.

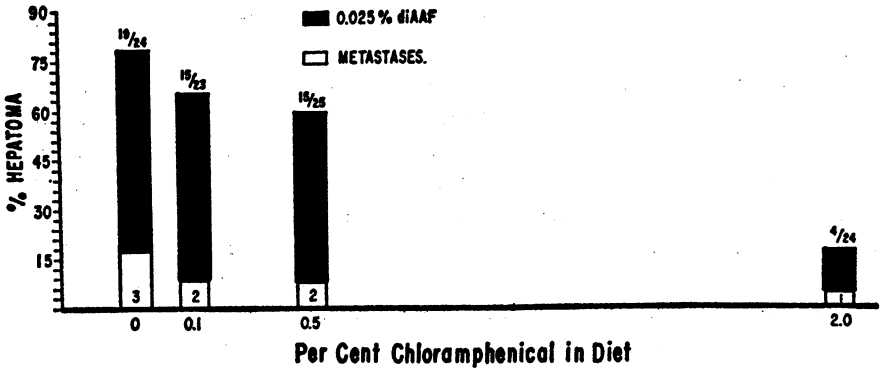

FIG. 2.

EXPERIMENT II

Plots of the absorption of the acidified liver protein extracted from the four groups of rats in Experiment II are shown in Figure 3.

The decrease in content of 554 mμ absorbing fluorenyl compound in the liver of the relatively immune female rats, compared to male rats, is clearly evident. After one week, a statistical analysis applying the standard "t" test to these two groups revealed a significance with a $p << 0.001$.

Male rats protected by the addition of 2% chloramphenicol to their diet showed some delay in obvious inhibition of binding but clearly diverged after three weeks from the male animals fed carcinogen only. The significance of decreased binding in the chloramphenicol group similarly has a p value of $<< 0.001$. In a separate experiment not included here, in which rats were fed a diet containing double the dose of carcinogen (0.05%) as was used in the long-term experiment reported previously,[1] entirely comparable inhibition occurred in the chloramphenicol-treated rats.

DISCUSSION

Previous studies had shown the procarcinogenic action of androgenic and anabolic hormones in this model.[2] It was known from the work of the Millers[4,5] and others[6-8] that the carcinogen was bound to protein in the liver. Although these hormonal effects might increase the net metabolic quantity of active proximate carcinogen in the liver, it seemed more likely that the effects of the anabolic hormones was through increased protein synthesis in the liver which bound more carcinogen as an integral component when protein intake was partially restricted.

The role of the anabolic hormones as described by Kochakian[9] was considered to be the induction of more protein synthetic machinery in the liver

cells, such as aspartic-glutamic transaminase, alanine-glutamic transaminase and d-amino acid oxidase. Reuber[10] showed the simultaneous need for thyroid hormone to maintain or increase the production rate by the synthetic machinery and presumably, thereby, the binding of carcinogen in the liver cells.

If protein synthesis is an integral part of the mechanism of carcinogenesis,

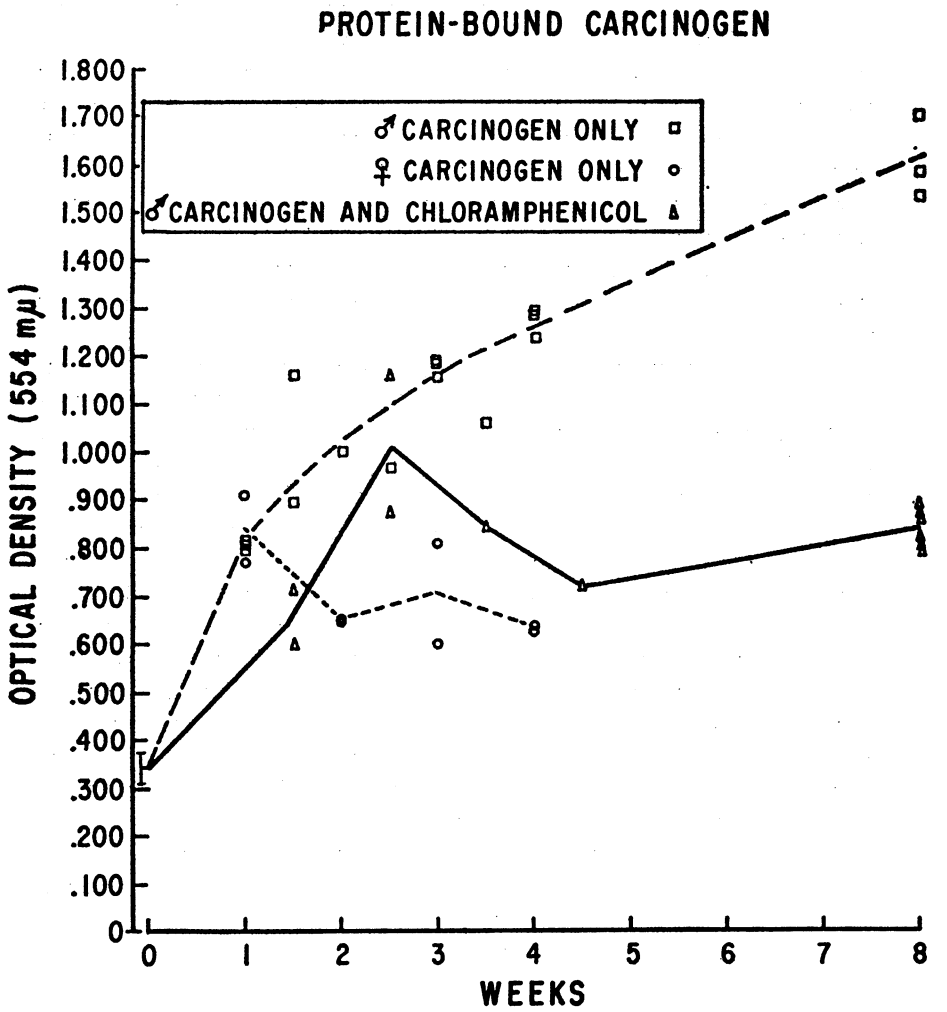

FIG. 3. Each point represents the determination from an individual animal. The zero time represents the average optical density of the extracts from 20 male rats fed the basic diet and killed periodically up to eight weeks after the start of the experiment. The range at zero time represents the 95% confidence limit for basic diet determinations.

perhaps an agent which is known to block protein synthesis in bacteria[11,12] in rabbit reticulocytes,[13] reproduction of normal human marrow cells and leukemic cells,[14] and synthesis of antibody in tissue culture[15] and yet which is not overly toxic for chronic experiments in rats might be anticarcinogenic. Preliminary experiments did show striking protection not only against hepatic carcinomas but also against cirrhosis.[1]

In the present experiments, cirrhosis of the liver was not seen at the time of autopsy. Previous studies[3] using serial biopsies revealed that cirrhosis is reversible on the 0.025% dosage of carcinogen and only the cysts do not disappear. In the present experiment, the number and severity of the cystic changes in the liver was inversely proportional to the dosage of chloramphenicol. It had been found previously that a higher dose of carcinogen increases the degree and extent of cirrhosis more than it does the number of tumors and the extent of involvement in the liver. This fact, together with the shorter duration of the previous preliminary experiment, would seem to account for the presence of cirrhosis in the unprotected controls in that study.

The protective effect of chloramphenicol against the induction of hepatocellular carcinoma was confirmed by the present experiment and appears clearly dose related, but the mechanism is still unknown. The preliminary study of fluorenyl content of extracted liver protein does indicate decreased binding after three weeks when chloramphenicol is fed with the carcinogen. If binding in the liver is important in carcinogenesis and its binding is merely prevented by chloramphenicol, then the carcinogen should be released and excreted in the urine. This seems to be borne out by the increased number of tumors outside the liver in the protected animals (8/24) on 2 percent chloramphenicol compared to the threefold smaller number (8/72) in the combined unprotected group and the two less-protected groups. These differences are even more striking when one considers the increase only of the ear duct and urinary tract tumors, usual products of the somewhat more powerful but less specific N-2-fluorenylacetamide.[16] This is further substantiated by Weisburger and his associates[17] who showed that the amount of the more carcinogenic N-hydroxy-N-2-fluorenylacetamide in the urine was increased in rats fed N-2-fluorenyldiacetamide and concurrent chloramphenicol. These findings exclude a block in formation of the proximate carcinogen or increased detoxication through inductive effects as the mechanism of protective action of chloramphenicol.

The slower rise of bound compound in chloramphenicol-fed animals (Figure 3) may reflect a short temporary inhibition of protein synthesis. The delayed fall of bound fluorenyl compound could suggest some inductive action may have to occur before the chloramphenicol is effective.

Weisburger and his associates[11] showed no reduction of incorporation of C^{14} labelled leucine into the protein pool of the liver in rats given chloramphenicol. Although this does not exclude possible inhibited production of a specific protein it would seem to point strongly to competition between chloramphenicol and the carcinogen as the mode of action of chloramphenicol. Of course the site of the competition, whether for enzyme, energy mechanics, t-RNA or direct binding sites in protein or nucleic acids, is a matter for further study.

SUMMARY

N-2-fluorenyldiacetamide-induced hepatic carcinogenesis in rats is effectively inhibited by feeding 2% chloramphenicol in the diet and is shown to be a dose-related phenomenon. Chloramphenicol effectively reduces binding of the carcinogen in the liver but does not prevent its metabolic formation or increase its detoxication. Instead it results in an increase in the types of tumors induced by the carcinogen in sites outside the liver. The findings suggest a competition between the carcinogen and chloramphenicol but the binding sites and the mechanism of their action requires further study.

REFERENCES

1. Puron, R. and Firminger, H. I.: Protection against induced cirrhosis and hepatocellular carcinoma in rats by chloramphenicol. *J. Nat. Cancer Inst.*, 1965, *35*, 29-37.
2. Firminger, H. I. and Reuber, M. D.: Influence of adrenocortical, androgenic and anabolic hormones on the development of carcinoma and cirrhosis of the liver in A X C rats fed N-2-fluorenyldiacetamide. *J. Nat. Cancer Inst.*, 1961, *27*, 559-595.
3. Cochran, W. G.: Some methods for strengthening the common X^2 tests. *Biometrics*, 1954, *4*, 417-451.
4. Miller, E. C. and Miller, J. A.: *In vivo* combinations between carcinogens and tissue constituents and their possible role in carcinogenesis. *Cancer Res.*, 1952, *12*, 547-556.
5. Miller, E. C. and Miller, J. A.: Biochemical investigations on hepatic carcinogenesis. *J. Natl. Cancer Inst.* (*Supp.*), 1955, *15*, 1571-1590.
6. Dyer, H. M. and Morris, H. P.: Studies on the protein binding of N-2-fluorenylacetamide. *J. Nat. Cancer Inst.*, 1956, *17*, 677-697.
7. Gutmann, H., Seal, U. S., and Irving, O. C.: On the mechanism of protein binding of N-2-fluorenylacetamide. The deacetylation of N-(1-hydroxy-2-fluorenyl) acetamide and the effect of ethionine. *Cancer Res.*, 1960, *20*, 1072-1078.
8. Weisburger, J. H., Grantham, P. H., Morris, H. P., and Weisburger, E. K.: On the *in vitro* and *in vivo* protein-binding of N-2-fluorenylacetamide and related compounds. *J. Nat. Cancer Inst.*, 1961, *27*, 153-171.
9. Kochakian, C. D.: Mechanisms of androgen actions. *Lab. Invest.*, 1959, *8*, 538-556.
10. Reuber, M. D.: Importance of thyroid hormone and testosterone in the induction of carcinoma and cirrhosis of the liver in female Wistar rats ingesting N-2-fluorenyldiacetamide. *J. Nat. Cancer Inst.*, 1966, *36*, 775-781.
11. Gale, E. F. and Folkes, J. P.: The assimilation of amino acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in Staphylococcus aureus. *Biochem. J.*, 1953, *53*, 493-498.

12. Tomizawa, J. and Sunakawa, S.: The effect of chloramphenicol on deoxyribonucleic acid synthesis and the development of resistance to ultraviolet irradiation in E. coli infected with bacteriophage T2. *J. gen. Physiol.*, 1956, *39*, 553-565.
13. Weisburger, A. S., Armentrout, S., and Wolfe, S.: Protein synthesis by reticulocyte ribosomes. I. Inhibition of polyuridylic acid-induced ribosomal protein synthesis by chloramphenicol. *Proc. nat. Acad. Sci. (Wash.)*, 1963, *50*, 86-93.
14. Yunis, A. A. and Harrington, W. J.: Patterns of inhibition by chloramphenicol of nucleic acid synthesis in human bone marrow and leukemic cells. *J. Lab. clin. Med.*, 1960, *56*, 331-383.
15. Ambrose, C. T. and Coons, A. H.: Studies on antibody production. VIII. The inhibitory effect of chloramphenicol on the synthesis of antibody in tissue culture. *J. exp. Med.*, 1963, *177*, 1075-1088.
16. Firminger, H. I.: Histopathology of carcinogenesis and tumors of the liver in rats. *J. Nat. Cancer Inst. (Supp.)*, 1955, *15*, 1427-1442.
17. Weisburger, J. H., Shirasu, Y., Grantham, P. H., and Weisburger, E. K.: Chloramphenicol, protein synthesis, and the metabolism of carcinogen N-2-fluorenyldiacetamide in rats. Inhibition by chloramphenicol of carcinogen binding. *J. biol. Chem.*, 1967, *242*, 372-378.