Nauts, Swift, and Coley (14) gave an exhaustive review of the clinical reports, and of animal experimentation, on the influence of bacterial toxins on malignant tumors, since the method was first used by W. B. Coley in 1892.

Beebe and Tracy (9) found that suspensions of Bacillus prodigiosus, Streptococcus pyogenes, Staphylococcus aureus, and B. coli communis caused regression of large transplanted lymphosarcomas in the dog. Similar results were obtained with bacterial preparations by Uhlenhuth, Haendel, and Steffenhagen (27) on rat tumors. Positive results were obtained only when the material was injected directly into the tumor. Gratia and Linz (11) found that B. coli filtrates produced hemorrhage and liquefaction in transplanted liposarcomas in the guinea pig.

Schwartzman and Michailovsky (21) reported the complete disappearance of tumors in mice after injecting meningococcus filtrate. Duran-Reynals (8) used Eberthella typhosus, B. enteriditis and B. paratyphosus filtrates and showed that in mice the newly formed tumor capillaries were very sensitive to these toxins. Apitz (1) studied the hemorrhagic reaction in 197 mouse carcinoma by means of B. coli filtrates and other bacterial substances and concluded that the walls of the tumor capillaries were damaged. This was not a generalized vascular reaction, but was confined to the tumor capillaries.

Shear began in 1933 to fractionate the hemorrhage-producing substance from B. prodigiosus and found it to be a polysaccharide. Following this he (22) studied the effect of meningococcus filtrate and various bacterial metabolites and found that in certain cases liquefaction and hemorrhage of the tumor occurred with an occasional complete regression. He (23) next separated the hemorrhage-producing fraction of the B. coli filtrate and produced extensive hemorrhage in mouse tumors. Severe hemorrhage was produced in subcutaneous primary induced mouse tumors by Shear (24) within a few hours after injecting the concentrated filtrate from B. prodigiosus in these tumors, but not in the normal tissue.

Shear (25) used doses as low as part of a microgram from the polysaccharide of B. prodigiosus on 750 mice bearing subcutaneous primary tumors, induced by 3,4 benzopyrene, and produced hemorrhage in them within a few hours. Diller (6) found that the polysaccharide of B. prodigiosus, when injected intravenously into a tumor-bearing animal, caused nuclear changes in the tumor cells. These changes involved surface blistering, shrinkage, pyknosis, and attenuation of nuclei leading ultimately to their complete destruction.

Of special bearing on our problem are the extensive studies by Morse and Scott (13) on histological changes produced in normal animals by the lipoid, protein, and polysaccharide fractions of the tubercle bacillus. Sabin and Doan (16) observed numerous cellular changes in rabbits after injecting the proteins and phosphatide fractions of human tubercle bacilli and in 1930 Sabin, Doan, and Forkner (17) reported further studies on the reaction of normal rabbit tissues to lipoid, protein, and polysaccharide fractions of Mycobacterium tuberculosis H37. Sabin et al. (18) found that the polysaccharide may have some killing power under certain conditions, but this is not as consistently related to dosage as in the case of proteins. Smithburn and Sabin (26) showed that the phosphatide fraction stimulates the formation of characteristic epithelioid and giant cells. Schaefer (20) separated the lipoid, polysaccharide, and protein fractions of tubercle bacilli and studied their action on normal mammalian tissues. Delaunay et al. (5) have demonstrated the leucocytic chemotactic power of certain constituents of tubercle bacilli in the guinea pig.

It is the purpose of the writers to describe the effect of the protein, phospho-lipoid, and polysaccharide fractions of Mycobacterium tuberculosis on methylcholanthrene-induced tumors in the rat.

MATERIALS AND METHODS

The tumors were induced by a single injection of 10 mg. of methylcholanthrene (Eastman Kodak Co., Rochester) dissolved in sterile olive oil. The rats were
injected subcutaneously in the left inguinal region and spindle-celled sarcomas appeared in between 4 and 8 months. Injections of the bacterial filtrates were started as soon as the tumor was visible macroscopically. The sex and weight of the tumor-bearing rats were recorded at the time we started injecting the bacterial extracts, which were made three times weekly. At the same time macroscopic observations of the tumor, the general appearance of the rat, and measurements of the tumor, were recorded.

Preparation of Mycobacterium tuberculosis extracts.
—Extracts were made from six to eight weeks old M. tuberculosis hominis cultured on the surface of Sauzan's synthetic medium at 37° C.

The phospho-lipoid, protein, and polysaccharide fractions were extracted according to the methods described by Schaefer (30).

To extract the phospho-lipoid fraction the cultures were first sterilized at 120° C. for 1 hour, passed through filter paper and washed on the filter with distilled water. The bacilli were then dried in a vacuum jar containing calcium chloride, weighted and treated with pure acetone for 24 to 36 hours at 25° C. (1 cc. acetone to 100 mg. of bacilli). The bacilli were filtered again, dried as before and placed in 99 per cent methyl alcohol for 10 days at 37° C. (1 cc. methyl alcohol per 100 mg. of bacilli). The amount of total phosphate in the filtrate was determined by the method of Fiske and Subbarrow (9). This methyl alcohol filtrate was diluted 1:10 with sterile normal saline (0.85 per cent) containing 0.5 per cent phenol and tubed into sterile vaccine bottles. The final concentration of the filtrate was 0.001 mg. per cc. The filtrate was stored in the refrigerator at 5° C. The yield of phosphates from the bacilli varied between 0.02 to 0.004 mg. per 1 gm. of dried tubercle bacilli, the average of 4 determinations being 0.012 mg.

To extract the protein fraction the living cultures were first passed through filter paper after which the filtrate was passed through a Seitz filter. To each liter of filtrate 600 gm. of ammonium sulfate was added in order to precipitate the proteins and the polysaccharides. After standing one hour the filtrate was centrifuged at 5000 r.p.m. for 20 minutes, the supernatant fluid was discarded, and the precipitate was dissolved in distilled water. In order to remove the ammonium sulfate the filtrate was next placed in a cellophane bag, containing thymol as preservative, and dried in vacuum over sulfuric acid. The dried material, which included the proteins and polysaccharide fractions of the bacilli, amounted to 0.5920 to 0.5972 per cent trichloroacetic acid (920 cc. acid to 100 cc. solution), and the precipitated proteins were separated by centrifugation. The supernatant fluid, which contains the polysaccharide, was decanted and saved. The precipitate, containing the proteins, was extracted with water and reprecipitated with trichloroacetic acid. This process was carried out three times, the supernatant fluid being retained in each case. After the third precipitation the protein precipitate was extracted in neutral distilled water and the extract was dialyzed for 24 hours at 5° C. This fraction was dried in a vacuum, dissolved in sterile saline (0.85 per cent) containing 0.5 per cent phenol, and tubed into sterile vaccine bottles. The final concentration of protein, 1.5 mg. per cc., was stored in the refrigerator at 5° C.

To extract the polysaccharide fraction the combined supernatant fluids obtained, as shown above, were treated with three volumes of 96 per cent ethyl alcohol, plus one volume of ether, in order to precipitate the polysaccharides. The precipitate was obtained by centrifugation and was washed twice with ether, and dried in vacuum over sulfuric acid. The dried polysaccharides were dissolved in sterile saline (0.85 per cent) containing 0.5 per cent phenol. The final concentration of the polysaccharides was 2 mg. per cc. The material was tubed into sterile vaccine bottles and stored in the refrigerator at 5° C.

Method of injection.—Into each of 10 tumor-bearing rats 0.001 mg. of the phospho-lipoid fraction was injected at the site of the tumor 3 times weekly. The minimum number of injections, before the death of the animal, was 5 and the maximum number was 15 (Fig. 1).

Thirteen tumor-bearing rats had 1 mg. of the protein fraction injected 8 times a week into the tumor. Three of the rats died after the second injection and two after the fifth, while 5 had from 8 to 11, and 3 had 14 injections (Fig. 2).

Into 11 tumor-bearing rats 1 mg. of the polysaccharide fraction was injected into the tumor 3 times a week. Two rats died after the seventh injection and the rest had from 9 to 14 injections (Fig. 3).
RESULTS AND OBSERVATIONS ON TUMOR-BEARING RATS

Phospho-lipoid fraction.—Ten rats with tumors were used in this series. On macroscopic observation of the tumor, bleeding was observed in most animals while introducing the needle after the third and on subsequent injections. The tumor became soft, congested, and extensively hemorrhagic. The condition of the animal became gradually worse, the tumor continued to increase in size and most of the rats died within about 4 weeks. Measurements were made of two diameters of the tumors at the time of each injection; however, the depth of the tumor could not be ascertained accurately and was not taken. These dimensions were multiplied to give surface values in square centimeters.

Figure 1 shows that the growth of the tumors followed more or less the same pattern with three deviations. Rat No. 10 showed no increase in the size of its tumor, while rats Nos. 1 and 3 had a much higher rate of growth than the average. An "average graph," based on the averages of the ordinates, is represented by the broken line.

Microscopic observations showed no evidence of cell destruction or of tumor regression following the injections that could be ascribed to the fraction (Figs. 4 and 5). There were, however, the usual areas of necrosis characteristic of a rapidly growing spindle cell sarcoma, but there was no lymphocytic infiltration.

Protein fraction.—Thirteen rats were used in this series. As in the case of the phospho-lipoid fraction, profuse hemorrhage was noted. There is more deviation in the tumor sizes of this group than in the preceding. Rats 4, 8, and 9 had much faster growth rates, while rats 6, 11, and 12 were quite sluggish; however, the "average graph" is comparable to the graphs for the other two fractions (Fig. 2).

Microscopically there was no evidence of more cellular destruction than the usual variations in untreated tumors.

Polysaccharide fraction.—In most of the eleven rats of this series, hemorrhage was indicated by softness of the tumor and the appearance of blood following the third or fourth injections.

More uniformity was encountered in the tumor growths of this group of rats. The tumor of rat 11 failed to grow and in this manner resembles rat 10 of group 1, and to a lesser extent rats 11 and 12 of group 2. The "average graph" is similar in shape and extent to the other two (Fig. 3).

The microscopic observations following the polysaccharide fraction were similar to those previously described. All the tumors were highly malignant, with no pattern of arrangement, and many multi-nucleated cells were present. The amount of blood vessels was variable, with areas of hemorrhage and necrosis.

RESULTS AND OBSERVATIONS ON CONTROL RATS

Phospho-lipoid fraction.—Three control rats each received a total of 14 injections of the phospho-lipoid (0.001 mg.) fraction subcutaneously in the left inguinal region. Bleeding was not apparent at any injection, in any of the controls, and all the
animals survived the treatment. After the four-
teenth injection the control rats, and some of the
tumor-bearing rats, were killed and both the left
and right inguinal areas were fixed in formalin,
sectioned in paraffin, and stained with hema-
toxylan and eosin.

Microscopically, distinct tubercles were ob-
erved at the site of the injections (Figs. 6 and 7)
with typical epithelioid and giant cells. The reac-
tion was in the connective tissues and muscle, but
the lymph nodes had no tubercles. There was an
intense diffuse mononuclear response and lympho-
cytes, fibroblasts, and giant cells were observed
in the region of the injection.

Protein.—Three rats were given 14 injections
(1 mg.) of the protein fraction. Slides made from
tissue in the region of the injections show a non-
specific foreign body reaction. There was a mono-
nuclear cell infiltration and giant cells; lympho-
cytes, and phagocytes were also observed.

Polysaccharide.—Three control rats received a
total of 14 injections (1 mg.) of this fraction. There
were lymphocytes, phagocytes, giant cells, and
many fibroblasts, but there was no specific reac-
tion in the injected area. The lymph nodes in the
region showed no tubercles, but diffuse mono-
nuclear cells were numerous.

DISCUSSION

In a series of preliminary studies we prepared
and injected a series of tumor-bearing rats with
Coley’s toxin, and the filtrates of E. typhosus and
S. paratyphosus. Our results were comparable to
those of Coley (4), Duran-Reynals (7), Shear (25),
and others.

Sabin and Doan (16) observed in normal rabbits
numerous cellular changes after the injection of
protein and phosphatide fractions of the human
tubercle bacilli. The phosphatide caused develop-
ment of monocytes, epithelioid cells, and Langhans
giant cells making a typical tuberculic lesion. Lymphocytic infiltration and some necrosis were
also noted. Their protein fractions caused multiple
capillary hemorrhages and an increase in debris
and bacilli filled clasmatoocytes. Sabin (19) also
found that the protein fraction induced the forma-
tion of monocytes in the normal animal, and
tubercles of epithelial cells in tuberculous animals.

After considering the findings of previous work-
ers, we chose to inject the fractions into the tumors
rather than intravenously or intraperitoneally. Beebe
and Tracy (2) and Uhlenhuth et al. (27) found
positive results only when their material
was injected into the tumor; furthermore dead
tubercle bacilli are toxic when injected in-
travenously or intraperitoneally.

The dosage we used was based on comparable
work of previous authors for other bacterial
products, but was near the higher rather than the
lower dosages. Long and Seibert (12) used
1/10,000 mg. of precipitated protein with am-
onium sulfate per guinea pig and Sabin (19)
gave 50 mg. of a similar fraction to each rabbit.
Our 1 mg. dosage given 3 times a week for a period
of 3 to 5 weeks seemed to be a plausible amount for
rats.

Our macroscopic findings agree fairly well with
those of previous workers in the presence of
hemorrhagic and softening areas; however, it
should be recalled that untreated tumors often
have such areas, and that our treated tumors were
not affected in a regressive manner. In spite of the
hemorrhage the growth curves showed that the
tumors continued to grow, more or less, in a uni-
form pattern and rate, in the three different filtrate
series, with a few exceptions which remain unex-
plained. The exceptional cases were rat No. 1 of
the phospho-lipoid series and rat 11 of the poly-
saccharide series whose tumors did not progress.
Some other rats had a much higher growth rate
than the average as shown in the graphs. Other
than these exceptions the growth curves in the
three series were very similar and there is no evi-
dence of one extract being more effective than
another.

Histologically we found that control rats, with-
out tumors, showed similar reactions to those de-
scribed in mice and rabbits by the earlier authors
quoted above, but to a less striking extent, due
perhaps to the higher resistance of the rat. Differ-
ence in resistance was shown by Gerstl and Ten-
nant (10) who found that the mouse is more re-
sistant than the rabbit, or the guinea pig, to ex-
tracts from tubercle bacilli and they attribute this
to its more rapid enzyme action. Pinner (15) also
states that rats are highly resistant to tubercle
bacilli and do not develop destructive tissue
processes.

We have observed that the phospho-lipoid frac-
tion caused in the normal rat distinct tubercles
with typical epithelioid and giant cells, with in-
filtration of monocytes, lymphocytes, and with
some necrotic areas. The polysaccharide fraction
produced streaks of white blood cells in addition to
other cellular elements at the site of injection in
the normal rat. After the protein fraction we found
in the normal rat a mononuclear cell infiltration.
Giant cells, phagocytes, and lymphocytes were
also observed; however it was a non-specific
foreign-body reaction.

In our tumor-bearing rats, on the other hand,
we failed to note such a tissue reaction as we de-
FIG. 4.—Section of tumor treated with phospho-lipoid fraction. Hematoxylin and eosin. ×140.
Fig. 5.—Same as Fig. 2. ×280.

FIG. 6.—Section from the site of injection of phospho-lipoid showing tubercle formation in control rat. Hematoxylin and eosin. ×140.
Fig. 7.—Same as Fig. 4. ×280.
scribed in the normal rats. We found no evidence of destructive changes due to the filtrates, for the phosphorylated and necrotic areas, such as we found, are usually present in a rapidly growing methylcholanthrene-induced tumor of the same kind, hence cannot be attributed to the filtrates themselves.

In conclusion, the three extracts of the tubercle bacilli used, which were capable of stimulating some tissue reaction in the normal rats, produced no destructive effects on the fibrosarcomas of the same animal. It might be worth while to repeat the work with an animal that is more susceptible to the tubercle bacillus.

**SUMMARY**

1. Three extracts of the tubercle bacillus: phosphoryl lipid, a protein, and polysaccharide were prepared.
2. Tumors were produced in rats by subcutaneous injections of methylcholanthrene.
3. Injections of the extracts were given into the tumors, the growth was measured, and histological studies were made. No evidence of destructive effect of the extract was noted.
4. Control rats were given, in the inguinal region, the same extracts. Histological studies revealed tissue reaction, as expected, with the appearance of typical tubercles with the phosphoryl lipid fraction.
5. It is suggested that a more susceptible animal than the rat should be used for the same work.

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Effect of Tubercle Bacilli Extracts on Induced Tumors of the Rat

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