of potassium from stilbestrol-treated cells was demonstrated.

1. Tateno, I., and Kilbourne, E. D., unpublished data.

2. Ponder, E., *Hemolysis and Related Phenomena*, 1948, Grune and Stratton.

Received April 30, 1954. P.S.E.B.M., 1954, v86.

Effects of Polyoxyethylene Sorbitan Monolaurate (Tween 20) upon Gastrointestinal Iron Absorption in Hamsters.* (21042)

ROBERT W. WISSLER, WILLIAM F. BETHARD,† PATRICIA BARKER, AND HIDEO D. MORI.

From the Departments of Pathology and Medicine, University of Chicago, and the Argonne Cancer Research Hospital, Chicago, Ill.

In this study iron[59] has been utilized to evaluate the effects of feeding polyoxyethylene sorbitan monolaurate (Tween 20) upon iron absorption. The experiments were performed because Eagle(1) observed that prolonged feeding of rations containing 5, 10 or 15% Tween 20 or other polyoxyethylene derivatives to golden hamsters resulted in consistent hemosiderosis of the cecum, liver and mesenteric lymph nodes and frequently in pigmentary cirrhosis.

This finding may be of some practical importance since polyoxyethylene and sorbitan compounds with fatty acids have been proposed as emulsifiers in many human foods. The theoretical implications are also evident, since investigation of the mechanism of development of these lesions may be helpful in understanding the pathogenesis of hemochromatosis and may increase knowledge of the factors regulating iron absorption from the gastrointestinal tract.

Methods and materials. Three experiments with similar plan have been performed. In each experiment adult hamsters of similar age and weight were divided into 2 groups. One group received a bread ration containing 5% Tween 20 while the second group was given a similar control ration without the Tween. In the first experiment the Tween 20 replaced some of the fat in the experimental ration while in the latter 2 experiments it was incorporated in the diet in addition to the lipid. The compositions of the rations, which were modeled after those described by Eagle are shown in Table I. The iron content of the salt mixture was such that each animal received at least 0.004 g of dietary iron (as ferric salts) per day. To prepare the rations the dry ingredients were mixed and the fat and/or Tween 20 was blended with them. The yeast was dispersed in the required amount of water and this was mixed with the other ingredients to make a dough. This was

TABLE I. Diet Compositions.

Ingredients	Tween ration Exp. 1 (g)	Tween ration Exp. 2 & 3 (g)	Control ration (g)
Vegetable fat	0	5	5
Tween 20	5	5	0
White flour	75.7	75.7	75.7
Baker's yeast	1.5	1.5	1.5
Salt mixture*	1	1	1
Lactalbumin	9	9	9
Sucrose	2	2	2
Water to make dough	56.6 ml	56.6 ml	56.6 ml
Added to ground bread			
Salt mixture*	3	3	3
Wheat germ oil	1	1	1
Vit. A & D oil	1	1	1
Vit. mixture†	0.8	0.8	0.8
Total	100	100	100

* Jones and Foster(2).

† Thiamin HCl 80 mg, riboflavin 80 mg, pyridoxine HCl 80 mg, calcium pantothenate 440 mg, niacin 400 mg, biotin 1.0 mg, folic acid 2.5 mg, 2-methyl 4-naphthoquinone 100 mg, choline chloride 10 g, para-aminobenzoic acid 1.0 g, inositol 1.5 g, Wilson 20:1, liver powder 70 g.

* This investigation was supported in part by Research Grants from the National Institutes of Health, Public Health Service.

† Work done during the tenure of a Damon Runyon Senior Clinical Research Fellowship of the American Cancer Society.

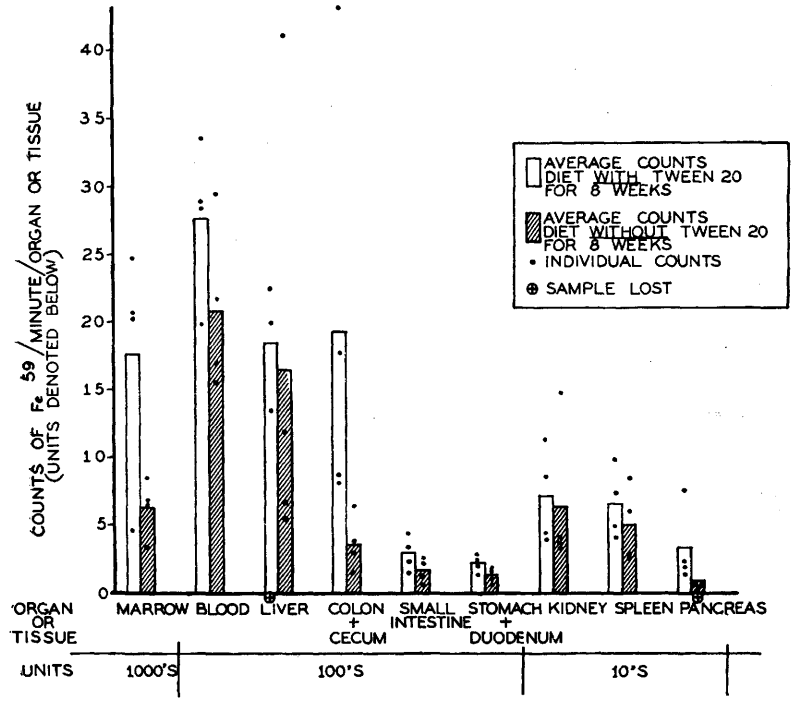

FIG. 1. Exp. 1. Summary of distribution of Fe^{59} in organs and tissues of Tween-fed and control hamsters 3 days after receiving 344000 counts of Fe^{59} by stomach tube. Individual and average counts are expressed as counts per min. per total organ or tissue and are corrected for decay.

allowed to incubate in a warm place for about 2 hours and was then baked at 200°C for 30 minutes. The resulting bread was sliced and air dried at room temperature. It was then ground and the remaining ingredients were mixed with the pulverized ration.

After receiving the rations for varying times equal groups of control and Tween fed hamsters were given concurrently an appropriate single dose of Fe^{59} by stomach tube.‡ The animals were then transferred to individual metabolism cages and urine and feces were collected daily. Seventy-two hours later the animals were sacrificed and specimens of various organs were obtained, weighed and prepared for counting as were the urine and fecal samples. In the first experiment carrier iron was added to each flask containing a sample to make a total of about 10 mg of iron. Wet ashing and electroplating of the sample were then carried out by the method of Peacock and Evans(3) as modified by Huff(4). The plated samples were counted using a lead-shielded end-window Geiger-Muller tube (Tracerlab TCG-2). The samples from the second and third experiments were counted directly in small plastic tubes using a well type scintillation counter. In the second and third experiment each animal was perfused with 100 ml of 0.86% NaCl before the tissue samples were taken to minimize blood contamination. Precautions were observed in each experiment to recover feces adhering to the cages. When residual radioactivity in carcasses was determined in Exp. 3, care was taken to include intestinal contents and fecal material about the anus in the excreta compartment. The parts of the intestinal tract were washed in iron free NaCl before counting. The carcasses were ground

‡ The Fe^{59} was supplied by Oak Ridge National Laboratory, and was prepared by neutron bombardment of electromagnetically separated Fe^{58}. Before use, the $FeCl_3$ solution was neutralized by addition of saturated sodium citrate with chlor-phenol red as an indicator.

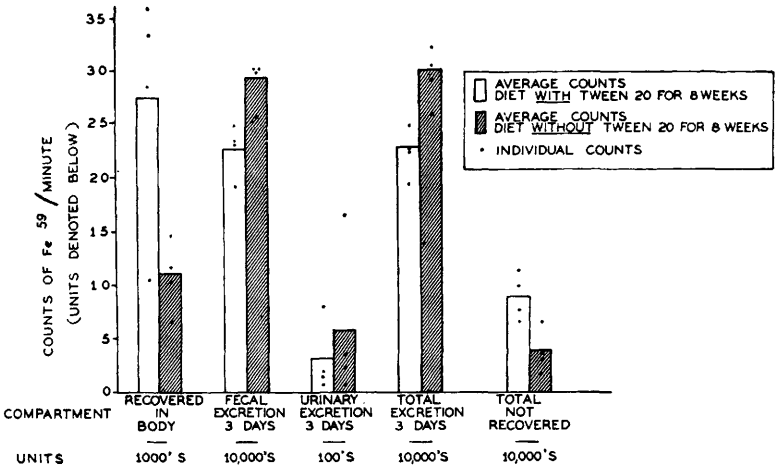

FIG. 2. Exp. 1. Summary of absorbed and excreted Fe^{59} in Tween-fed and control hamsters 3 days after receiving 344000 counts of Fe^{59} by stomach tube. Results are corrected for decay.

in a meat grinder, dissolved in 2 N NaOH, heated at 60°C for 2-3 days, and aliquots were then removed for counting. Samples of liver, spleen and cecum were wet ashed and analyzed for total iron along with appropriate controls in Exp. 3 by the method of Barkan and Walker(5) with ascorbic acid substituted for hydrazine sulfate as the reducing agent.

Small tissue samples from each animal were fixed in formalin, imbedded in paraffin and stained for iron by the Prussian blue reaction. Hematoxylin and eosin stains were also examined from the liver, spleen, pancreas, kidneys, mesenteric lymph nodes and the various parts of the gastrointestinal tract.

Results. Diet consumption and weight gains of the 2 groups of hamsters were essentially the same in each experiment. Intermittent diarrhea was apparent in both groups but was more common in the Tween fed animals. Fig. 1 summarizes the individual and average radioactivity measured in Exp. 1 from the tissues of 4 pairs of hamsters 3 days after each animal had been given Fe^{59}, equivalent to 344000 counts per minute or about one μc and about 1.5 μg, and after they had received the respective diets for 8 weeks. Although considerable individual variation was encountered, there was a definite trend for the animals receiving the Tween to show more radioactivity in the marrow, blood, liver and large intestine 3 days after the test dose of Fe^{59} had been given. In each instance the counts represent the calculated amount of radioactivity in the total organ or tissue.§

Fig. 2 demonstrates that the greater amount of Fe^{59} apparent in the tissues of the Tween fed animals was associated with a decreased excretion of Fe^{59} and that there was more unrecovered in the Tween fed animals than in the controls, possibly due to excess Fe^{59} present in the parts of the carcass not analyzed or due to incomplete recovery of excreted Fe^{59}.

Although the daily fecal and urinary Fe^{59} excretion have not been illustrated, individual results indicated that the majority of the Fe^{59} was excreted during the first 48 hours. It is noteworthy that very little of the Fe^{59} was excreted in the urine, an observation which agrees with previous studies of iron metabolism(6,7).

In the second experiment 2 groups of animals were studied after they had been fed the respective rations for 10 weeks. The experiment differed from the first in that the Tween was added to the ration rather than being substituted for a portion of the fat in the ration, the tissues were perfused before samples were obtained for counting and the

§ For these calculations the theoretical total bone marrow was considered to be 1%(4) of the body weight and blood was considered to be 5% of the body weight.

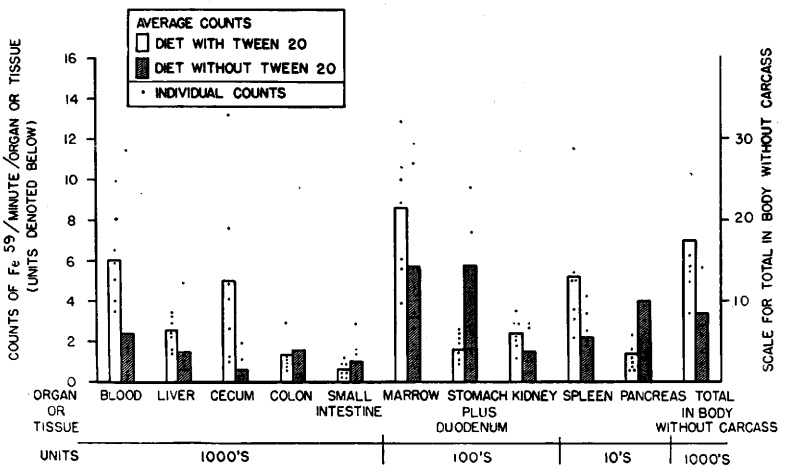

FIG. 3. Exp. 2. Summary of individual and average counts of Fe^{59} in organs and tissues of Tween-fed and control hamsters 3 days after receiving 375000 counts of Fe^{59} by stomach tube. Results are corrected for decay and are expressed as counts per min. per total organ or tissue.

more direct gamma counting methods were employed. Each animal received 2 ml of the iron solution containing 375000 counts per minute or approximately 0.5 μc in about 1.0 μg of iron, and each was sacrificed 72 hours later. Fig. 3 illustrates the same trends noted in the first experiment. The blood, marrow, liver and cecum of the Tween fed animals contained more of the ingested dose than the same organs of the control animals. Two factors complicated this experiment: 1) occasional control animals showed unusually high Fe^{59} values which detracted from the statistical competence of the data. 2) It was impossible to obtain a good measure of carcass Fe^{59} since adherence of fecal material about the anus caused some of the residual carcass counts to be very high and the excreta counts to be correspondingly low. Measures to avoid this complication were taken in the third experiment.

The third experiment was similar to the second except that the animals had been fed the respective rations for 20 weeks before the tracer dose of about one million counts of Fe^{59} containing about 1.0 μc and about 1.5 μg of iron was administered orally. In this experiment only the organs or tissues which had shown consistent differences in the first 2 experiments as well as the spleens were counted separately. A measure of the remaining radioactivity in the animal was obtained by determining the counts on the residual carcass. In Fig. 4 it is apparent that with one or 2 exceptions quite consistent differences were observed between the organs of the Tween fed animals and those of the control animals. The cecum showed the greatest contrast between the 2 groups; the cecums of the Tween fed animals averaging almost 40 times the amount of Fe^{59} as compared to the cecums of the control animals. The other organs and tissues of the Tween fed hamsters tested averaged about 2 times the quantity of Fe^{59} found in the controls. Similarly the residual carcasses of the animals receiving Tween contained about 6 times as much Fe^{59} as the control carcasses. The overall balance of absorbed and excreted Fe^{59} is also shown in this chart. Here again it is apparent that there was a definite difference between the 2 groups with the Tween fed animals showing more absorption and less excretion. The group receiving Tween 20 had an average of about 8% of the ingested Fe^{59} unaccounted for in the overall balance, whereas there was no such deficit in the control group. It seems probable that most of this deficit was due to incomplete recovery of excreted Fe^{59} in 2 of the Tween fed animals. A small part of the deficit was undoubtedly due to the samples of the cecum and other organs removed for histological study, since these samples constituted a larger proportion of the ingested tracer dose in the hamsters

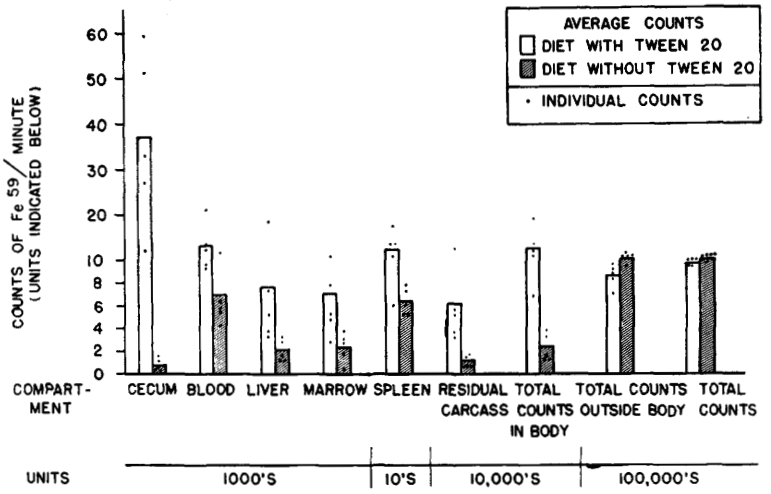

FIG. 4. Exp. 3. Summary of individual and average counts of Fe^{59} in certain organs and in other compartments 3 days after Tween-fed and control hamsters received 1 million counts of Fe^{59} by stomach tube. Results are corrected for decay and are expressed as counts per min. per total organ or tissue or per total excreta.

fed the Tween ration. In any case addition of these missing counts on either side of the balance sheet would not alter the conclusions to be drawn.

Hemoglobin and hematocrit determinations on the blood of each of the animals of this experiment at the time of sacrifice demonstrated no anemia and no differences between the 2 groups. Therefore anemia does not appear to have caused the increased iron absorption in the Tween fed group.

Histological study of the Prussian blue stained tissues of the 2 groups of animals in each experiment revealed a consistent increase in stainable iron in the cecums, large intestines, livers, and mesenteric lymph nodes of the animals receiving Tween 20.

Furthermore, total iron analyses on the spleens, livers, and cecums of the animals of Exp. 3 revealed an average of about 5 times as much iron in the cecums of the Tween fed animals as in the cecums of the controls and about 1½ as much in the livers of the Tween fed animals as in the livers of the controls. There was no significant difference in the iron content of the spleens of the 2 groups (Fig. 5).

The stainable iron in the cecums was found in the epithelial cells and in the macrophages of the stroma of the mucosa (Fig. 6). The increased visible hepatic iron was largely in parenchymal cells about the portal triads with lesser quantities in the Kupffer cells and in the macrophages of the portal triads (Fig. 7). Occasional livers of the Tween fed groups of hamsters showed early cirrhosis (Fig. 8) but the marked cirrhosis accompanying the iron deposition reported by Eagle(1) in younger hamsters receiving polyoxyethylene derivatives for longer periods of time was not observed. No appreciable inflammation of the gastrointestinal mucosa was observed

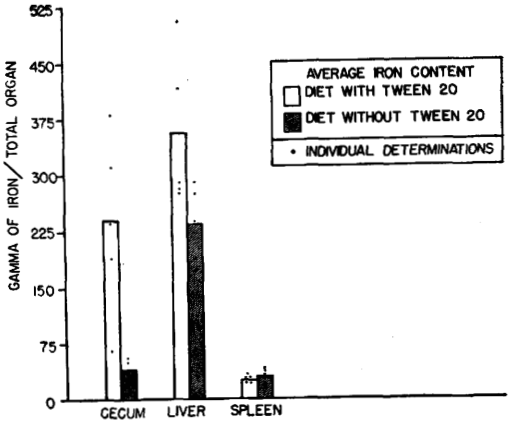

FIG. 5. Exp. 3. Results of chemical analysis of selected organs for total iron expressed as gamma of iron per total organ.

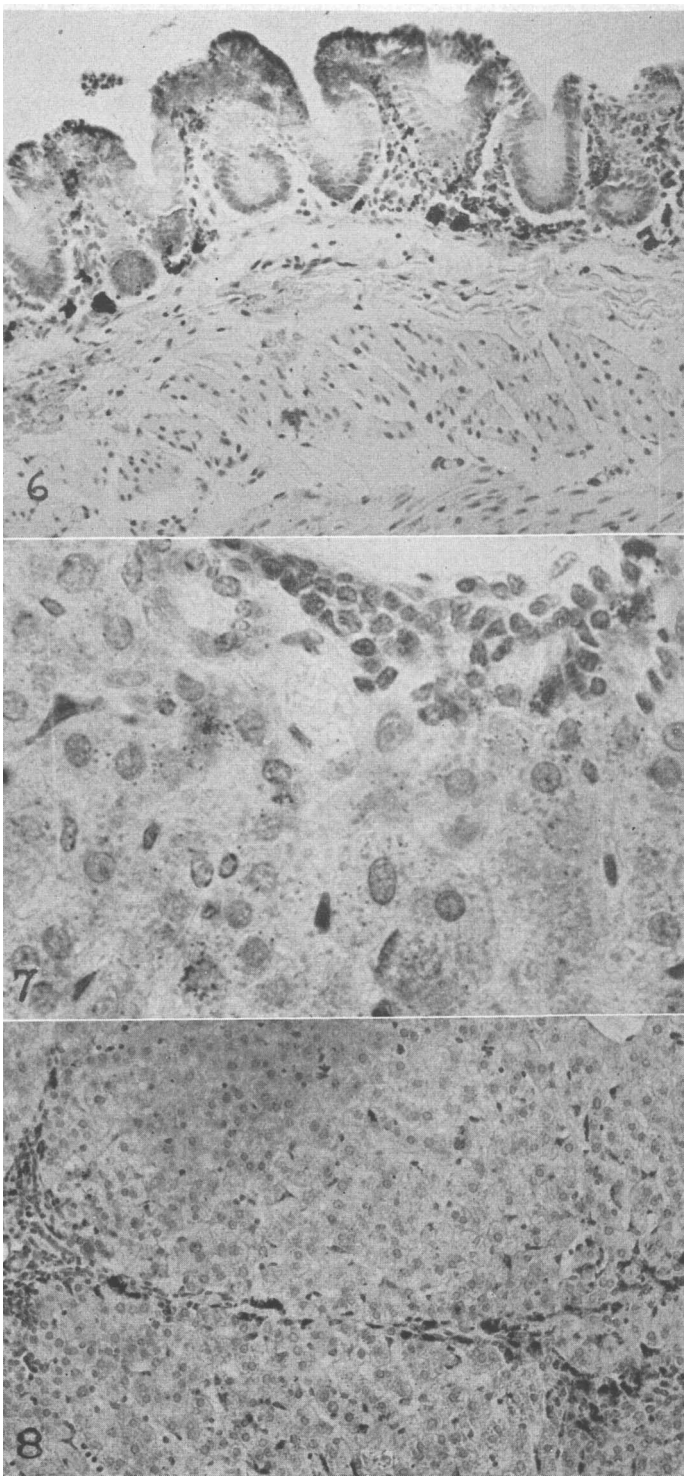

FIG. 6. Cecum of hamster from Exp. 3, showing diffuse accumulation of iron in epithelial cells nearest the lumen and in macrophages in stroma of the mucosa. ×193.

FIG. 7. Liver of hamster from Exp. 3 stained by Prussian blue reaction. Dark granules in the parenchymal cells, Kupffer cells and periportal macrophages represent stained iron. ×634.

FIG. 8. Liver of hamster from Exp. 3 showing early cirrhosis and abundant iron positive granules in macrophages of the fibrous tissue and in the Kupffer cells. ×150.

in any of the animals receiving the Tween ration.

Comment. The results of these 3 experiments indicate that the presence of 5% Tween 20 in the diet of the golden hamster results in a consistent increase in the gastrointestinal absorption of iron. This apparently increases with the time the animal is maintained on the Tween containing ration. It occurs when the Tween 20 is added to the ration or when it is substituted for a part of the fat in the ration. It is accompanied by an increase in histologically demonstrable iron in the cecum and to a lesser extent the rest of the large intestine as well as the liver and mesenteric lymph nodes. The increase in hepatic and cecal iron has also been demonstrated by chemical analysis. The absence of anemia in the Tween fed hamsters indicates that this well known mechanism(7) for increasing iron absorption from the gastrointestinal tract was not responsible for the differences between the 2 groups.

The largest and most consistent differences in Fe^{59} content between the experimental and control animal tissues 3 days following the administration of the tracer dose of iron by stomach tube occurs in the gastrointestinal tract, bone marrow and blood. This is noteworthy since these are the tissues concerned in iron absorption, erythrocyte formation and erythrocyte circulation.

Although the mechanism by which Tween 20 increases iron absorption from the gastrointestinal tract is not yet clear, the results of this study would suggest that the excess iron is being absorbed largely in the cecum. The cecums enlarge in the Tween fed animals and their contents become more fluid. Associated with this is a tendency toward diarrhea, but no inflammation of any part of the gastrointestinal tract was observed histologically in the Tween fed animals. The similar weight gains and diet consumption of the 2 diet groups in these relatively short feeding periods fail to implicate an inherent toxicity of the 5% Tween ration or any gross malnutrition in the mechanism. Whether the emulsifying agent acts mainly on the epithelium to increase its permeability or whether it converts gastrointestinal iron into a more absorbable physical or chemical state remains to be investigated.

The results of the experiments suggest but do not prove that one mechanism by which the usually well-regulated gastrointestinal absorption of iron can be increased is by extending the area of absorption from the small intestine, where the majority is usually absorbed(7-9), to the large intestine.

Summary. In 3 experiments adult golden hamsters fed a fortified bread ration containing 5% polyoxyethylene sorbitan monolaurate (Tween 20) for from 8-20 weeks showed a consistent increase in gastrointestinal absorption of a test dose of radioactive iron (Fe^{59}) during the subsequent 3 days. Increases in the isotope were noted especially in the cecum and large intestine, the blood, bone marrow, and liver. Concomitantly less radioactive iron appeared in the excreta of the Tween fed animals than in the excreta of control animals. This evidence of increased iron absorption in the hamsters was accompanied by the presence of iron pigment in the cecal mucosa, mesenteric lymph nodes and liver and by an increase in chemically demonstrable iron in the cecum and liver.

The authors wish to acknowledge the technical assistance of Mrs. Marjorie Schroeder and Mrs. Kathryn Soules. The photomicrographs were taken by Mr. Jean Crunelle, chief photographer, University of Chicago Clinics.

1. Eagle, E., *et al.*, Testimony at Hearings before the House Select Committee to Investigate the Use of Chemicals in Food Products. Union Calendar No. 1139, Rep. 3254, 81st Congress, 2nd session, Nov. 16, 1950, pp. 296 ff. U. S. Govt. Printing Office, Washington, D.C., 1951.

2. Jones, J. H., and Foster, C., *J. Nutrition,* 1942, v24, 245.

3. Peacock, W. C., Evans, R. D., Irving, J. W., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J. G., *J. Clin. Invest.*, 1946, v25, 605.

4. Huff, R. L., Bethard, W. F., Garcia, J. F., Roberts, B. M., Jacobson, L. O., and Lawrence, J. H., *J. Lab. and Clin. Med.,* 1950, v36, 40.

5. Barkan, G., and Walker, B. S., *J. Biol. Chem.,* 1940, v37, 1935.

6. McCance, R. A., and Widdoson, E. M., *Lancet,* 1937, v2, 680.

7. Vannotti, A., and Delachaux, A., *Iron Metabolism,* Grune & Stratton, 1949.

8. Hahn, P. F., Bale, W. F., Hettig, R. A., Kamen, M. D., and Whipple, G. H., *J. Exp. Med.*, 1939, v70, 443.

9. Robscheit-Robbins, F. S., *Physiol. Rev.*, 1929, v9, 666.

Received May 3, 1954. P.S.E.B.M., 1954, v86.

Intradermal Test for Determining Resistance to Infectious Canine Hepatitis Virus.* (21043)

J. E. PRIER AND S. S. KALTER.

From the Department of Microbiology, State University of New York at Syracuse.

Studies on infectious canine hepatitis virus (ICH) are limited, in part, because of a) the inability to use a convenient small laboratory animal and b) complement-fixation tests are not always a reliable index of past infection. Many resistant dogs may show a negative complement-fixation test. The costly and elaborate procedure for raising disease-free animals is impractical for most laboratories. Street dogs are barred from routine use because of natural infection and resulting immunity.

In the course of clinical and experimental studies with ICH virus, it was found that the intradermal injection of apparently normal dogs with lymphoid tissue from an infected animal caused a marked reaction in some cases. The relationship of this reaction to resistance to ICH virus was, therefore, investigated.

Materials and methods. Virus strains. Two strains of infectious canine hepatitis virus were used in the study. One strain, designated 9868, was obtained from Dr. H. R. Cox and the other, 21V33, from Dr. C. J. York. Both strains were passed through at least 2 serial passages in dogs before use. Inocula used were 30% suspensions of infective liver. Intravenous injections were used and dogs killed on the third post-inoculation day by an overdose of sodium pentabarbital. Several dogs were used for each passage and one chosen that demonstrated a sharp temperature rise and simultaneous clinical symptoms of the disease. The liver was harvested under sterile conditions and placed in a previously weighed Waring blendor flask. The flask and liver were weighed and the weight of the liver alone determined. Sufficient buffered saline (pH 7.2) was added to make a 30% suspension of liver and the mixture homogenized for 3 minutes. The homogenized material was distributed in 2 ml amounts to sterile ampoules, sealed, shell frozen and stored in a dry ice chamber. Prior to intravenous injection, an additional 1 ml of sterile saline was added to the thawed suspension. *Street dogs* obtained from a stock supply were used without regard to age, size or sex. The great variation in animals led to the assumption that this sampling would represent a cross section of an average urban canine population. One hundred and twenty-four dogs were used in the series. Dogs were maintained in community pens except for challenge experiments, when they were placed in individual cages. *Complement-fixation tests.* A liver antigen was used in all tests. This was prepared from the liver of a dog experimentally infected with strain 9868 (Lederle). The liver was homogenized with sufficient buffered saline to make a 30% suspension. The mixture was centrifuged at 2000 r.p.m. for 15 minutes. The supernatant was distributed to 1 liter round-bottom flasks and shell frozen in a dry ice-alcohol bath. After immediate thawing, the mixture was recentrifuged at 2000 r.p.m. for 30 minutes. The resulting supernatant was distributed in 50 ml amounts, formalized (0.4%) and shell frozen. Lyophilization was performed after storage for 24-72 hours at −20°C. The dried material was ground with a mortar and pestle and stored in brown bottles at 4°C. This material

* Aided in part by a grant from the Hendrick's Research Fund.