thermoregulatory center is depressed or because not enough circulating pyrogen reaches the center to activate it. Conversely, some animals may develop fever in the absence of EP, as was the case with leukopenic animals given relatively small amounts of endotoxin. The dissociation between EP and fever has been attributed by Gillman, Bornstein and Wood to an insufficient quantity of EP in the circulation to demonstrate by passive transfer(11). With this technique the pyrogen would be diluted in a large volume of blood and no fever would be evident. These authors argue that enough pyrogen is present in the serum of the donor, however, to activate the thermoregulatory center. It is unlikely, however, that this explanation can be invoked in the present experiments because both normal and leukopenic animals had the same amount of fever when given the same relatively low dose of endotoxin. In one instant EP was present, in the other it was not. It is clear that the magnitude of the febrile response was within the sensitive part of the dose-response curve to endotoxin (12) and was not in the hyperthermic range in which alterations in fever would be difficult to demonstrate. It is more likely that under certain restricted circumstances toxic substances such as nitrogen mustard or even endotoxin *per se* alter the blood-brain barrier (13), permitting leakage of endotoxin into the CNS. Once in the spinal fluid, the toxin may act directly on the thermoregulatory centers to produce fever(14). These studies are not presented as an argument against the hypothesis that endotoxin or other types of fever are mediated by endogenous pyrogen. However, they emphasize again that fever may occur in the absence of this substance.

Summary. In normal rabbits given endotoxin, the degree of pyrexia and release of EP are proportional to the dose of endotoxin injected, until very high dosages are reached, at which point hypothermia occurs and the animals die. In leukopenic rabbits the hypothermic and lethal effects are evident at much lower dosage. These animals produce no endogenous pyrogen. At the lowest doses tested leukopenic animals had as much fever as normals but did not release EP.

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Determination of Avidin by Use of Radioactive Biotin and Sephadex Chromatography.* (29485)

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Microbiological methods(1) have long been used for determination of avidin, the

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protein which produces egg white injury by combining irreversibly with biotin(2). These methods are extremely sensitive, but are quite time-consuming. Furthermore, in view of the extraordinary stability of the avidin-biotin complex towards heat, independently found in this laboratory(3), and by Pai and Lichstein(4), the results obtained from microbiological methods become questionable. In addition to microbiological methods, Launer and Fraenkel-Conrat(5) first used radioactive biotin for study of avidin-biotin equilibrium by a dialysis technique. Recently, Green(6) developed a rapid and simple method for assay of avidin based on the determination of the excess of radioactive biotin after adsorption of the avidin-biotin complex on carboxymethylcellulose. A spectrophotometric method of greater accuracy but lower sensitivity, based on a biotin-induced spectral shift, has also been presented by Green(7). In this laboratory, for the purpose of studying the behavior of the avidin-biotin complex, a method of assay for avidin also has been developed. The principle of the method is to add an excess of radioactive biotin to samples of avidin solution, and an aliquot of this mixture is then passed through a column of Sephadex G-25. The excess biotin (small molecule) is retarded on entering the pores of the Sephadex gel, while the avidin-biotin complex (high molecular weight molecule) is confined to the liquid medium exterior to the gel proper. This gives a 2-peak chromatogram, based on the radioactivity of the separate fractions, namely the avidin-biotin complex in the first peak and the excess biotin in the second peak. The size of the avidin-biotin peak is a measure of the avidin present in the original solution.

Materials and methods. D-biotin-carboxyl-C^{14}, with a specific activity of 22.9 mc/millimole was kindly provided by Dr. O. Wiss, Hoffmann-LaRoche, Basel, Switzerland. D-biotin-carbonyl-C^{14}, with a specific activity of 3.56 mc/millimole was purchased from New England Nuclear Corp, Boston, Mass.

Avidin was purchased from Nutritional Biochemicals Co. (N.B.C.), Cleveland, Ohio, with an indicated activity of 2.5 units/mg. Pure avidin also was prepared by a new method of Melamed and Green (M. & G.) (8) with an activity of 13.1 units/mg.

Sephadex G-25, Medium, was obtained from Pharmacia, Uppsala, Sweden. It had a water regain of 2.5 g/g of dry gel. Before use, the fines were removed by several decantations. Two column volumes of developing solution, in these experiments 0.2 M ammonium carbonate, were passed through the packed column before use. The dimensions of the column were 0.8 cm $\times$ 33 cm.

Known amounts of about 0.4 unit of avidin in 1 ml 0.2 M ammonium carbonate and about 1 μg of D-biotin-carboxyl-C^{14} in 5 ml 0.2 M ammonium carbonate were mixed in a 10 ml volumetric flask. The flask was shaken gently for 15 minutes and then diluted to 10 ml with 0.2 M ammonium carbonate. An aliquot of 1 ml of this reaction mixture was applied to the Sephadex G-25 column. The column was then eluted by 0.2 M ammonium carbonate. Fractions of 6 drops (0.575 ml) were collected directly in 20 ml scintillation counting vials placed on an automatic fraction collector with a drop counting unit (Research Specialties Co.). Ten ml of Bray's scintillation mixture(9) was added to each vial, and the vials were counted by means of a Packard Tricarb-Scintillation spectrometer operating at an efficiency of 36%.

Results. A representative experiment demonstrating the determination of avidin by radioactive biotin is shown in Fig. 1. One ml of the reaction mixture containing 0.0026 mg of pure avidin (M. & G.) and 0.13 μg (10,000 cpm) of D-biotin-carboxyl-C^{14} was chromatographed on the Sephadex G-25 column. The area of the first peak was equivalent to 2710 cpm, corresponding to 0.036 μg of biotin. This is in excellent agreement with the calculated result, $0.0026 \times 13.8 = 0.036$ μg, based on 1 mg of pure avidin combining with 13.8 μg of biotin(8). From the amount of biotin present in the first peak, in turn the amount of avidin can be calculated.

To ascertain whether or not the separation of avidin-biotin complex and the excess free biotin is complete, 2 experiments were con-

TABLE I. Avidin-Biotin and Biotin Recoveries for Varying Quantities of Avidin with a Constant Quantity of Biotin.

Exp No.	Amt of avidin (N.B.C.), mg	Amt of biotin, cpm	1st Peak, avidin-biotin, cpm	2nd Peak, excess biotin, cpm	Total recovery, cpm	% of total recovery
1	.021	10000	2040	7900	9940	99.4
2	2 × .021	10000	4270	5440	9710	97.1
3	4 × .021	10000	8420	1440	9860	98.6
4	0	10000	0	10020	10020	100.2

ducted. Fig. 2 shows an increased amount of avidin (M. & G.) (up to 0.35 mg was employed in a single experiment) which was used with an excess amount of D-biotin-carbonyl-C^{14} of a lower specific activity (3.56 mc/millimole) in order to trace the radioactivity of biotin and the OD at 280 mμ of avidin on a chromatogram at the same time. The result in Fig. 2 indicates that no protein was found in the second peak. The results of another approach are summarized in Table I. Varying the amount of avidin (N.B.C.) present in the reaction mixture, with a constant amount of biotin, it was found that the area of the first peak was directly proportional to the amount of avidin present. In the absence of avidin, as is shown in Exp. 4, there was no biotin in the region of the first peak. As indicated in Exp. 1, 0.021 mg of avidin (N.B.C.), equivalent to 0.0525 unit of avidin, was used, and was expected to give 3900 cpm in the first peak. The lower value of 2040 cpm, probably due to partial denaturation of the protein during storage or the use of a different procedure of assay by Nutritional Biochemicals Co. However this is irrelevant for the purposes of this experiment.

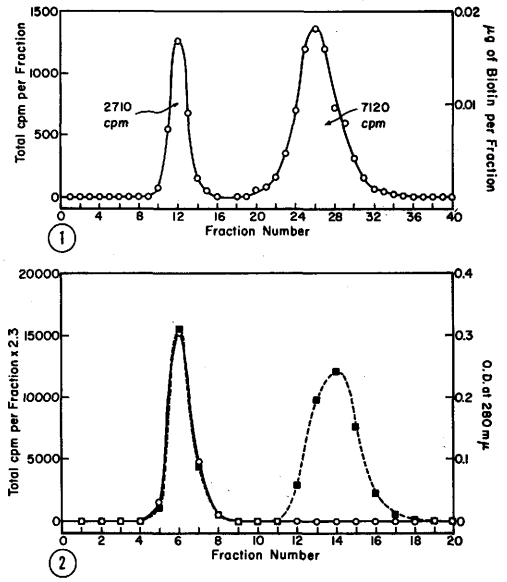

FIG. 1. Gel-filtration of 1 ml of avidin and biotin reaction mixture on a column (0.8 cm × 33 cm) of Sephadex G-25 in 0.2 M ammonium carbonate. Fractions of 6 drops (0.575 ml)/vial were collected.

FIG. 2. Gel-filtration of 1 ml of 0.35 mg avidin and excess of D-biotin-carbonyl-C^{14} reaction mixture on a column (0.8 cm × 33 cm) of Sephadex G-25 in 0.2 M ammonium carbonate. Fractions of 12 drops (1.15 ml) were collected and the OD at 280 mμ were measured on Beckman DU Spectrophotometer. From each fraction 0.5 ml was taken for radioactivity measurement. O——O OD at 280 mμ, ■——■ radioactivity.

TABLE II. Effect of Temperature During Determination of Avidin.

Temp, °C	1st Peak, avidin-biotin, cpm	2nd Peak, excess biotin, cpm	Total recovery, cpm
3	1670	7260	8930
30	1810	7080	8890
60	1790	6900	8690

Table II shows the determination of avidin carried out at different temperatures. The first peak, representing the avidin-biotin complex, does not vary significantly in the range of 3°C to 60°C.

Discussion. The use of 0.2 M ammonium carbonate, as a solvent and as a developing solution throughout the experiments, was initially based on the dialysis experiment of Launer and Fraenkel-Conrat(5). This proved fortuitous since the ionic strength of 0.2 M ammonium carbonate would appear to be optimal for the formation of the avidin-biotin complex(3). The importance of the ionic strength of the solution in which avidin and biotin are allowed to combine(3) and dissociate(4) have been emphasized recently.

The results of the study of the effects of temperature during the determinations indicated that the avidin-biotin complex is relativley stable with respect to heat. Further investigations of the stability of avidin-biotin complex at higher temperatures will be reported from this laboratory(3).

Summary. A method for determination of avidin by use of radioactive biotin and a gel-filtration technique has been developed. The method is based on measurement of the radioactivity of the avidin-biotin complex which separates from free biotin when a reaction solution of avidin and excess radioactive biotin is applied to a Sephadex G-25 column.

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Influence of Autoimmune Hemolytic Anemia on Susceptibility to *Salmonella* Infection.* (29486)

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Previous studies(1,2) have shown that intramuscular injection of phenylhydrazine hydrochloride or intravenous administration of anti-mouse-erythrocyte serum, heterologous erythrocytes or antibody-coated homologous erythrocytes markedly increases the susceptibility of mice to infection caused by *Salmonella typhimurium, Escherichia coli* or *Staphylococcus aureus*. The hypothesis has been advanced that phagocytosis of heterologous or altered homologous erythrocytes by reticuloendothelial cells impairs the capacity of these cells to kill ingested bacteria.

The present studies were undertaken to investigate the effect of naturally-occurring autoimmune hemolytic anemia on susceptibility to *Salmonella* infection utilizing the NZB/Bl strain of mice. This strain was developed by Bielschowsky by successive brother-sister matings(3) and has been studied by Holmes and Burnet(4). The NZB/Bl mice begin to develop autoimmune hemolytic anemia after 13 weeks of age.

Methods. Four female and 2 male litter mates of the 48th generation of the NZB/Bl strain were generously supplied by Sir Macfarlane Burnet and Dr. Margaret C. Holmes in March, 1962. By successive brother-sister matings a colony was developed. The studies to be reported were performed on mice of the 49th through 53rd generations (continuing Bielschowsky's numbering system).

Hematocrit values were determined by a micro-hematocrit method(1) in heparinized capillary tubes, 75 mm in length and 1.2 to 1.4 mm in diameter. Approximately 0.03 ml of blood was collected from the retro-orbital venous plexus and centrifuged for 3 minutes at 12,500 rpm in a microhematocrit centrifuge (Clay-Adams, Inc.). Hematocrit values were read directly from the tubes using a micro-capillary reader (International Equipment Co.).

Sera used in performing Coombs tests were prepared in rabbits. Two parts of pooled mouse serum from male Swiss mice of the

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