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Persistence of C^{14}-Anthranil-azo-ovalbumin in Injected Rabbits.* (19653)

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The organ deposition and intracellular distribution of heavily radio-iodinated antigens following their injection into rabbits has already been reported(1-4). Iodine 131 suffers from certain disadvantages as an antigen label in *in vivo* experiments. Iodoproteins, due to their similarity to the thyroid hormone, could conceivably be metabolized in a manner not representative of antigens in general. Moreover, the short half-life of I^{131} renders experiments of very long duration impossible. Both of these difficulties were avoided in the present investigation by coupling the protein antigen with diazotized 7-C^{14}-anthranilic acid.

*Experimental. Preparation of 7-C^{14}-anthranil-azo-ovalbumin (A*AO).* Crystalline ovalbumin was prepared from hen's eggs by the method of Kekwick and Cannan(5). All operations for the preparation of A*AO were carried out in the cold, using chilled solutions. In a typical preparation, 33 mg 7-C^{14}-anthranilic acid,§ containing 27 μc per mg, was dissolved in 2.7 ml of 0.2 *N* HCl, and diazotized by adding 0.05 *N* sodium nitrite (4.5 ml) until there developed a positive test with starch and iodide. The solution was poured into 7.5 ml of a solution containing 3.3% sodium carbonate and 3.9% ovalbumin. Coupling was accelerated by the subsequent addition of 0.95 ml *N* NaOH. After standing over night, the solution was dialyzed against distilled water, precipitated by dilute hydrochloric acid, centrifuged, and the precipitated azoprotein suspended in water and dissolved by adding sodium carbonate to pH 7. A large amount of a non-radioactive preparation was prepared parallel with the radioactive one. The 2 preparations were mixed in various ratios to afford solutions for injection of the desired specific activity. Spectrophotometric comparison of our anthranil-azo-ovalbumin with anthranil-azo-tyrosine at a wave length of 460 mμ indicated 11.8 azo groups per protein molecule, assuming a molecular weight of

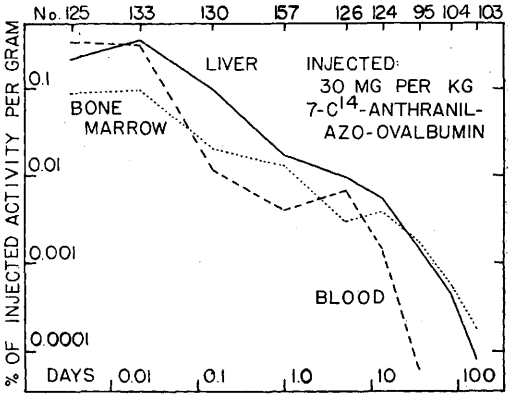

FIG. 1. Antigen content of blood, liver, and bone marrow after the injection of C^{14}-anthranil-azo-ovalbumin to rabbits. The number of each rabbit is recorded at the top of the figure; the vertical lines under the numbers indicate the time of killing; the time scale, at the bottom of the figure, is logarithmic. Analyses of other organs and of subcellular fractions of the same rabbits are recorded in Table I.

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TABLE I. Organ Levels and Intracellular Distribution in Tissue Proteins Following a Single Intravenous Injection of 30 mg/kg 7-C^{14}-anthranil-azo-ovalbumin (A*–Ov).

Rabbit No.	125	133	130	157	126	124	95	104	103
Time killed	5 min	30 min	3.5 hr	23.7 hr	4.7 d	11.8 d	33 d	79 d	148 d
Wt (kg)	1.6	2.2	2.2	1.5	1.9	1.8	2.9	2.9	2
Liver wt (g)	89	83	110	82	75	84	109	77	99
A*–Ov (%)†	18.2	28	10.7	1.35	.83	.50	.185	.040	.0073
Organ:	% of inj. activity/g whole tissue × 1000:								
Spleen	233	300	238	24.8	27.4	15.2	2.95	.90	.39
Lung	1780	139	26.5	3.06	8.41	1.64	.18	.096	—
Kidney	82	91	20.6	12.5	4.3	4.5	2	.6	.2
Heart	48	55	11	1.6	(10?)	1.2	.05	—	—
Liver fraction:	Wt of TCA-precipitates derived from a g of whole liver (mg):								
Nuclear	23.7	42.5	21.1	30.1	32.1	53.5	27.4	44.6	28.2
Mitochondrial	27.1	25.5	22.2	24.5	27	29.9	23.7	25.1	24.6
Microsomal	20.3	18.5	20.3	22.2	22.6	30.6	29.1	27.4	21.1
Supernatant	44.8	65.2	39.2	41.5	47.3	71	63.8	67.8	—
Liver fraction:	Relative activities:*								
Nuclear	1.49	.80	.87	1.60	1.07	.91	1.43	1.12	.82
Mitochondrial	1.45	3.05	2.57	1.77	1.84	2.18	3.65	3.40	4.1
Microsomal	1.46	1.31	.72	.38	.45	.67	.17	.71	2.3
Supernatant	.22	.22	.29	.51	.73	.61	.16	.15	.0
Spleen fraction:	Relative activities:*								
Nuclear	.54	.59	.66	.78	.58	.89	1.19	1.30	1.71
Mitochondrial	2.94	3.72	4.23	2.38	2.21	2.80	2.69	2.64	1.18
Microsomal	3.68	1.38	.89	1.53	3.45	1.12	1.12	.29	.0
Supernatant	.45	.44	.18	.72	.76	.42	.42	.12	.0

* Activity of total homogenate ≡ 1.
† In % of injected radioactivity per whole liver.

45000 for ovalbumin. Recovery of anthranilic acid radioactivity in the azoprotein indicated 11.9 residues per protein molecule.

Preparation of samples and counting of radioactivity. The experimental procedures with regard to sacrifice of the rabbits and the preparation and centrifugal fractionation of the organ homogenates were essentially those already described in detail(4). All injections were made intravenously into the marginal ear vein and consisted of 30 mg A*AO per kg of body weight. The livers of the rabbits were perfused with about 500 ml of chilled isotonic sucrose (0.25 *M*) via the portal vein. Homogenates were prepared using 0.88 *M* sucrose solution. The bone marrow was taken from the femur. Organ homogenates, fraction suspensions, and standard preparations were precipitated with trichloroacetic acid, and the precipitates washed as described previously (4). Radioactivity was measured in a Q-gas flow counter, or with a thin mica window Geiger tube. To correct for self-absorption, the counting rate of each sample was recalculated for a sample thickness of 8.1 mg/cm^2, using a calibration curve in which the activity of a C^{14}-containing protein precipitate was plotted against the sample thickness.

Results. Persistence of C^{14}-anthranil-azo-ovalbumin in organs. Fig. 1 and Table I give the data for organ deposition of trichloroacetic acid precipitable radioactivity following the injection of A*AO. In Fig. 1, the percentage of injected radioactivity per g of whole liver and bone marrow, and per ml of blood is plotted on a logarithmic scale against the time elapsing between injection and sacrifice, also plotted on a logarithmic scale. Similarly, tabulated data for lung, kidney, and spleen are given in Table I.

Intracellular distribution of deposited anthranil-azo-ovalbumin in liver and spleen. Table I also describes the distribution of radioactivity among the trichloroacetic acid precipitates of the subcellular fractions of liver and spleen, isolated from homogenates of these organs by differential centrifugation. The relative activities for liver were obtained by dividing the counts per minute per mg protein of a fraction, by the counts per minute per mg of the whole homogenate protein. The relative activity of the whole homogenate is

therefore 1.0, and the value for the several fractions tell how many times more concentrated the radioactivity is in the proteins of a particular fraction than in the proteins of the whole homogenate. The values for spleen were obtained by dividing the percent of the radioactivity residing in each spleen fraction, by the percent of the total spleen nitrogen it accounts for. The average spleen nitrogen distribution figures previously used(4) were employed.

Discussion. The results obtained here with C^{14}-anthranil-azo-ovalbumin in general parallel those obtained with I^{131}-iodo-ovalbumin(4) and arsanil-azo-proteins(6). From Fig. 1 and Table I it is apparent that the antigen is cleared rapidly from the blood, and that it is detectable in spleen, bone marrow, liver and kidney for several months. After long periods of time it is most concentrated in spleen and bone marrow. The persistence of anthranil-azo-ovalbumin in rabbit organs is in agreement with the long-term storage of azoproteins in the tissues of mice reported by McMaster and Kruse(7).

When injected *in vivo,* the antigen undergoes a brief initial concentration in the microsomal fraction of liver and spleen (Table I), and possibly of other organs as well. After a short time, however, the antigen is found chiefly in the mitochondrial fraction, where it appears to remain concentrated over long periods of time (Table I). The accumulation of the antigen in the cytoplasmic granules is not simply a non-specific adsorption effect; when the chilled liver or spleen homogenates from normal rabbits are mixed *in vitro* with the antigen, and 30 minutes later fractionated, or when the mixture of antigen and homogenate is incubated at 37°C for 15 minutes, the bulk of the radioactivity is recovered in the final supernatant (Table II, Nos. 140 and 151). The same result was obtained when homogenate No. 151 was incubated with the added antigen at 37°C for 30 or 45 minutes before beginning the fractionation.

In previous experiments with arsanil-azo-protein there remained the possibility that a portion of the arsenic found in the tissues was metabolized inorganic arsenic(6). The present experiments are free from this objection since we measured only trichloroacetic acid precipitable radioactive carbon.

TABLE II. Relative Activities of the Proteins of Subcellular Fractions after Addition of 7-C^{14}-anthranil-azo-ovalbumin *In vitro* to Homogenates of Normal Rabbit Organs.

Rabbit No.	140	140	151
Organ	Liver	Spleen	Liver
Antigen added (μg/g tissue)	95	119	95
Incubation: time (min)	30	30	15
temp. (°C)	<5	<5	37
Relative activity:			
Nuclei	.47	.31	.61
Mitochondria	.19	.32	.28
Microsomes	.28	.45	.32
Supernatant	1.86	2.75	1.68

The question arises as to whether all of the protein-bound C^{14} is present as unchanged antigen, or whether some of the radioactive anthranilic acid or anthranil-azo-tyrosine was split off and was recombined with insoluble tissue proteins. Although the formation of free anthranilic acid from anthranil-azo-ovalbumin and its incorporation into other proteins is highly improbable, we investigated this possibility by injecting into a rabbit (No. 156) C^{14}-anthranilic acid in an amount equivalent to 30 mg anthranil-azo-ovalbumin per kg body weight. The animal was killed after 24.5 hours. The radioactivity of the liver and blood proteins was 0.00016 and 0.00023% of the injected activity per g liver or ml blood, respectively; evidently, injected anthranilic acid was not deposited in the liver. Analysis of the urine of this animal showed almost quantitative excretion of the injected radioactivity through the kidney. When an equivalent amount of C^{14}-anthranil-azo-ovalbumin was injected into rabbit No. 157, 0.0165% of the injected radioactivity was found in one g liver, *i.e.,* 100 times more than after the injection of anthranilic acid. These results render unlikely the metabolic incorporation of C^{14}-anthranilic acid, split off from the injected azoprotein, as an explanation for the lengthy persistence of C^{14} in the tissue proteins. This is in accordance with observations of Kruse and McMaster(8) who have shown that free azo dye, in contrast to azo-protein, is not deposited in the organs, but is rapidly excreted.

Since the radioactivity of our antigen mole-

cules is localized in the determinant *o*-azobenzoic acid groups which are responsible for the serological specificity, our analyses reveal only the fate of these groups, not that of the ovalbumin moiety which, in our experiments, acts only as non-specific carrier of the determinant groups. Although the transfer of radioactive anthranil-azo-peptides from deposited antigen molecules to the proteins of the body cells is rendered unlikely by the experiments mentioned in the preceding paragraph, we cannot exclude this possibility. However, it is well known that low-molecular "proantigens" such as acyl chlorides(9) or oxazolones(10) are converted into true antigens by combination with proteins of the injected animal. The formation of anthranil-azo-tissue proteins would not affect, therefore, our view that the antigen, or, more precisely, its serologically active specific group, is stored in the body cells over long periods of time.

The question may be raised, whether the radioactivity of the tissue proteins is not due to the metabolic incorporation of radioactive carbon dioxide derived from the breakdown of anthranilic acid residues of the antigen. This is unlikely for the following reasons: I) After 24 hours, less than 10% of the injected radioactivity remains in the rabbit; the small residual amount of antigen is slowly degraded, and any carbon dioxide derived from it is diluted with a large excess of non-radioactive carbonic acid from the bicarbonate pool of the body; since most of the carbonic acid is excreted through the lung and the urine, only traces would be available for incorporation into body constituents. II) The ratio (C^{14} in heart muscle protein)/(C^{14} in liver protein) decreases in our experiments from a value of 0.2 on the 12th day to 0.03 on the 33rd day after injection. If $C^{14}O_2$ were incorporated into the tissue proteins, one would expect the opposite phenomenon because the turnover of muscle protein is slower than that of liver protein. This was first demonstrated by Shemin and Rittenberg(11) who observed an increase in the ratio (N^{15} in muscle protein)/(N^{15} in liver protein) during the incorporation of N^{15}-glycine.

We conclude from all these considerations that formation of $C^{14}O_2$ and its incorporation into tissue proteins play no significant role in our experiments and that most of the radioactivity found in the tissue proteins after long periods of time, is present as 7-C^{14}-anthranil-azo-protein. Although the persistence of the antigen in the tissue does not constitute definitive proof that the presence of the antigen is necessary for antibody formation, our results support this view. They are difficult to reconcile with theories based on the assumption that antigen disappears rapidly from the organs and that antibody formation continues after elimination of all of the antigen(12).

Summary. At various intervals of time following a single intravenous injection of 7-C^{14}-anthranil-azo-ovalbumin to rabbits, the organ deposition and intracellular distribution in liver and spleen of trichloroacetic acid precipitable radioactivity was determined. The antigen was cleared rapidly from the blood and deposited in various organs, where significant amounts were retained for many months; in the initial moments of deposition, the antigen was concentrated in the microsomal proteins of liver and spleen; subsequently it was most concentrated in the mitochondrial proteins, where it remained stored indefinitely.

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