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The Use of Radioactive Isotopes in Immunological Investigations

2. THE FATE OF INJECTED ^{32}P -CONTAINING PROTEINS

By T. E. BANKS, J. C. BOURSNELL (Beit Memorial Research Fellow), HILARY M. DEWEY, G. E. FRANCIS, R. TUPPER AND A. WORMALL, *Departments of Biochemistry and Physics, Medical College of St Bartholomew's Hospital, London*

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Isotopic tracers can be of considerable value in immunochemical investigations. ^{15}N has been used, for example, in studies on the formation and circulation of antibodies (Schoenheimer, Heidelberger, Rittenberg & Ratner, 1941; Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942). Radioactive isotopes of P and S have been used to determine the amount of tracer-containing antigen in antigen-antibody precipitates (Bournsnel, Dewey, Francis & Wormall, 1947), and in investigations on the lipid P and vitellin P in lipovitellin-antilipovitellin precipitates (Francis & Wormall, 1948). Libby (1947) and Libby & Madison (1947) have recently used ^{32}P -containing tobacco mosaic virus to study the rate of breakdown of this antigen in the animal body. There are obviously many other ways in which labelled antigens and antibodies can be used in the field of immunology.

In the present investigation, a preliminary study has been made of the fate of injected ^{32}P -containing antigens, phosphorylated human serum proteins and vitellin, to determine the length of time these antigens remain in the blood stream after injection.

METHODS

Phosphorylated proteins

Preparation of phosphoryl chloride containing ^{32}P . Radioactive sodium phosphate was diluted with ordinary phosphate and precipitated as $\text{Ca}_3(\text{PO}_4)_2$ (0.9 g.). This was dried and intimately ground with 0.9 g. of HCl-extracted charcoal (Merck). The mixture was thoroughly dried at 150° and then heated in a dry all-glass Pyrex apparatus in a metal bath at 300° , with dry air drawn through the $\text{Ca}_3(\text{PO}_4)_2$ -charcoal mixture to remove traces of moisture. A weighed receiver was connected and cooled in a CCl_4 -solid CO_2 mixture at about -25° and dry COCl_2 was passed through the mixture at 300 – 360° until the reaction ceased. The residual $\text{Ca}_3(\text{PO}_4)_2$ -charcoal mixture was removed and finely ground to break up the CaCl_2 layer which had formed over the remaining $\text{Ca}_3(\text{PO}_4)_2$; it was then reheated in a current of dry air and COCl_2 passed through as before. When this reaction had ceased, the POCl_3 in the receiver was warmed to remove COCl_2 and distilled. (Yield 0.44 g.; 49% of theory.)

Preparation of phosphorylated human serum proteins. Human serum (6.5 ml.) was phosphorylated with 0.229 g. of $^{32}\text{POCl}_3$ (i.e. a POCl_3 /protein ratio of 1:2) as described previously (Bournsnel, Dewey & Wormall, 1948). In this

experiment the proteins were not separated by precipitation with acid, but the solution was dialyzed at about 4° against 0.9% (w/v) NaCl until free from dialyzable phosphate; this product, which had a P/N ratio of 0.085, was treated with 0.05 vol. of 5% (w/v) phenol and used for injection; a similar preparation used for the precipitin tests had a P/N ratio of 0.065.

Treatment of the blood samples

Three rabbits each received an intravenous injection of ³²P-containing phosphorylated human serum proteins, and blood samples (a little over 5.5 ml. at each bleeding) were taken at intervals from the ear not used for the injection. A radioactivity determination was made on 0.50 ml. of each blood sample, and 5.0 ml. were centrifuged and the separated cells and plasma treated as follows:

Cell fraction. The cells were washed three times with 3 ml. of 0.9% NaCl each time, and a radioactivity determination was made on the whole sample.

Plasma. A radioactivity determination was made on 0.50 ml. of the plasma, and the remainder was used for separation of the proteins as follows:

(a) *Ethanol-ether precipitation.* Plasma (1.0 ml.) was added to 6 ml. of cold ethanol-ether (7:3 v/v) mixture, kept at 0° for 2 hr. and then centrifuged. The precipitate was washed twice with 6 ml. of cold ethanol-ether mixture each time, and three times with 6 ml. of cold ether. It was then Soxhlet-extracted with two 100 ml. portions of CaCl₂-dried ether for 2 hr. each time, and radioactivity determinations were made on the dried product. Determinations made on a few of the ethanol-ether extracts showed that these contained no significant amount of ³²P.

(b) *Trichloroacetic acid precipitation.* Plasma (1.0 ml.) was added to 4.0 ml. of cold 10% (w/v) trichloroacetic acid. After 2 hr. at 4° the precipitated proteins were centrifuged and washed three times with 3 ml. of 8% (w/v) trichloroacetic acid each time. Radioactivity determinations were made on the dried proteins.

Determination of radioactivity

The samples were incinerated with HNO₃, Cu and NaClO₃ as described previously (Boursnell, Francis & Wormall, 1946a). The resulting pyrophosphate was hydrolyzed by boiling for 10 min. with approx. N-HCl, and KH₂PO₄ equivalent to 100 mg. of Mg(NH₄)PO₄ was then added. The phosphate was separated as ammonium phosphomolybdate, and converted to Mg(NH₄)PO₄; this was dried at 105–110° and a weighed amount transferred to a ring (1.7 cm. diameter) mounted on a brass plate. The precipitate was spread evenly and protected by a few drops of dilute collodion solution in ethanol-ether. Radioactivity determinations were carried out as described for ³²S by Boursnell *et al.* (1946a), using the counter system described by Banks (1946).

Lipovitellin and vitellin

Preparation of antigens

Lipovitellin. A solution of radio-lipovitellin in 10% (w/v) NaCl was prepared from eggs laid by hens injected with ³²P-containing neutralized sodium phosphate, as described previously (Francis & Wormall, 1948).

Vitellin. A solution of radio-lipovitellin (c. 6% in 10% NaCl) was poured into 5 vol. of ethanol-ether (7:3) mixture at –15°. The mixture was centrifuged at once and the precipitate was washed once with ethanol-ether and twice with ether, all operations being carried out at between –15 and –5° and as rapidly as possible. Most of the adhering ether was removed by rapid evacuation and the protein was washed once with ice-cold water; it was then dissolved in a minimal amount of dilute NaOH and precipitated with dilute acetic acid, or dissolved in saturated urea solution and dialyzed. The products were emulsified with 0.9% NaCl solution with the addition of a few drops of 0.1N-NaOH and strained through muslin to give a fine cream suitable for injection.

Preparation of materials for radioactivity determinations

Blood. Blood from the marginal vein of the ear not used for the injection was collected in tubes containing oxalate at intervals after the injection of lipovitellin. The rabbits injected with vitellin died about 1 min. after the injection and in these cases blood was then taken from the heart as quickly as possible. Whole blood samples (3 or 5 ml.), or the separated plasma and cells, were evaporated to dryness several times with conc. HNO₃ (2–3 ml.) in 50 ml. flasks until a clear straw-coloured liquid was obtained. A further evaporation with HNO₃ plus 1–2 ml. of 10% (w/v) NaClO₃ completed the destruction of all organic matter.

Tissues. The whole organs (liver, spleen, heart, lungs and kidneys) were removed immediately after the death of the rabbits injected with vitellin; these organs were dried with filter paper, weighed, dried at 100–150° and ashed by heating in a muffle furnace for about 6 hr. at 400° and a further 6 hr. at 700–800°.

Radioactivity determinations. Preliminary experiments showed that where the residue from the incineration of a sample was less than 100 mg., accurate radioactivity determinations could be made without separating the phosphate; these residues were dissolved in water containing a few drops of 2N-HCl and the solution evaporated directly on copper or nickel holders. With larger residues, the material was dissolved in a mixture of N-HCl and N-HNO₃ (occasional insoluble fractions having been found to have no detectable radioactivity), and the solution boiled to hydrolyze pyrophosphoric acid. The phosphate was then precipitated with magnesia mixture, and a sample (up to 100 mg.) spread on a nickel holder for radioactivity determination. All the results recorded in the tables are average values for two or more determinations.

RESULTS

Injection of phosphorylated proteins

The serum proteins are strongly antigenic and, since phosphorylation can readily be effected at low temperature and at about pH 8, it was thought that phosphorylated human serum proteins might be suitable for immunological investigations. They are relatively non-toxic and can be injected in amounts sufficient to permit one to trace the antigen after its injection. The ³²P available for some of our experiments was rather small, but the radioactivity of the

phosphorylated proteins used here was high enough to allow reliable determinations to be made on small blood samples taken from the rabbits at intervals. In this experiment the mixed phosphorylated proteins were freed from inorganic phosphate by dialysis, but were not otherwise purified or separated, in order to minimize as far as possible the chemical changes effected in the protein antigen.

The results of an experiment with three rabbits showed (Table 1) that the injected antigen did not remain long in the blood stream. Assuming complete mixing of the injected material with the circulating blood, and assuming a blood volume of 50 ml./kg. of body wt. (the approximate average for the blood volume calculated from values quoted by Erlanger (1921) for the rabbit), the radio-P content of the blood of the injected animals would be about 8.6 $\mu\text{g./ml.}$ shortly after the injection. The average values observed were 6.4 $\mu\text{g.}$ after about 10 min., 2.0 $\mu\text{g.}$ after 3 hr., and 0.5 $\mu\text{g.}$ of radio-P/ml. of whole blood 24 hr. after the injection. As expected, the greater part of the ^{32}P was present in the plasma in the early stages of the experiment; the cell fraction of the blood contained only a very small amount 10 min. and 3 hr. after the injection. Determinations were not made on the cell fractions of later blood samples, since it was thought that any ^{32}P present in these cells might have originated from inorganic phosphate removed from the phosphorylated proteins. It will be observed that the sum of the radio-P contents of the plasma (adjusted on the basis of a plasma volume of 55–60 % of the whole blood volume) and the blood cell fraction does not quite agree in most cases with the value for whole blood. This might have been due to the loss of ^{32}P -containing compounds during the washing of the cells.

In some cases ^{32}P determinations were made on the proteins separated from the plasma samples either by treatment with ethanol-ether or by precipitation with trichloroacetic acid, with the object of finding out how much of the blood or plasma ^{32}P was present in the proteins. The values obtained with these separated proteins were similar to the

corresponding plasma values recorded in Table 1, and these plasma radio-P values can therefore be regarded as a measure of the amount of phosphorylated human serum protein present. In other investigations it has been found (Bournsnel *et al.* 1948) that treatment with ethanol-ether under the conditions of our experiments leads to precipitation of inorganic phosphate with the protein, but this does not appear to be a complicating factor here; the amount of radioactive inorganic phosphate liberated in the first few minutes and possibly even during the first 3 hr. after the injection would probably be small, and in any case much of it would readily diffuse from the blood to the tissues.

In an attempt to obtain, by a method independent of radioactivity determinations, an approximate measure of the length of time the injected protein, or its dephosphorylated counterpart, remained in the blood, some of the plasma samples were tested with antisera to human serum proteins. Since our antisera to phosphorylated proteins were not specific for the phosphate-containing groups of phosphorylated proteins, it was decided that serological tests should be made for the human serum protein part of the molecule. The results showed that significant amounts of human serum proteins, or their phosphorylated derivatives (since these are precipitated by antisera to human serum proteins) were present in circulation up to at least 5 days after the injection.

Injection of lipovitellin and vitellin

In view of the fact that the phosphate groups of phosphorylated proteins are fairly readily removed at 37°, injection experiments have been carried out with the more stable labelled antigens, lipovitellin and vitellin. Both compounds can be synthesized biologically and obtained after a moderate amount of chemical treatment and manipulation.

The results of experiments with lipovitellin showed (Table 2) that the injected compound rapidly left the blood; about 12 % only remained in the blood after 5 min., 2–4 % after 5 hr. and about 2 % 24 hr. after the injection.

Table 1. *Radio-P content of whole blood, cells and plasma of rabbits after the injection of phosphorylated human serum*

(Three rabbits *A*, *B* and *C*, weighing 3.25, 2.95 and 2.95 kg. respectively, each received an intravenous injection of 1.25 ml./kg. of a solution of dialyzed phosphorylated human serum containing 346 $\mu\text{g.}$ radio-P/ml. and 0.13 microcuries of ^{32}P /ml.)

Time after injection (hr.)	Radio-P content ($\mu\text{g./ml.}$ of whole blood or plasma, or in the cells from 1 ml. of whole blood)								
	Whole blood			Cells (washed)			Plasma		
	<i>A</i>	<i>B</i>	<i>C</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>A</i>	<i>B</i>	<i>C</i>
0.17	5.3	6.8	7.0	0.07	0.03	0.03	7.7	10.2	—
3	1.7	2.2	2.1	0.06	0.08	0.13	2.3	2.4	3.1
24	0.4	0.4	0.6	—	—	—	0.3	0.4	0.5
96	0.2	0.2	0.2	—	—	—	0.0	0.0	0.1
144	0.1	0.2	0.1	—	—	—	0.0	0.0	0.0

Table 2. *Radio-P content of blood of rabbits following the intravenous injection of ³²P-containing lipovitellin*

(Rabbits nos. 541, 544 and 529, of wt. 2.4, 2.4 and 2.3 kg. respectively, each received an injection of 3.0 ml. of lipovitellin solution containing 4572 μ g. of radio-P. Rabbit no. 541 received a further similar injection 11 days later.)

Rabbit no.	Time after injection (hr.)	Radio-P content (μ g./ml. of blood)			Total* radio-P in the blood of the rabbit, as % of that injected
		Whole blood	Plasma	Cells	
541	0.08	4.2	3.6	1.3	11
	5	1.3	0.6	0.7	3.5
	24	0.7	<0.15	<0.6	2
	48	1.4	<0.2	0.7	3.5
541 (2nd injection)	0.25	2.7	—	—	7
	4	<1.2	—	—	<3
	24	0.9	—	—	2.5
544	0.08	4.9	3.5	—	13
529	c. 0.08	5.0	3.4	—	13

* Assuming a blood volume of 50 ml./kg. of body wt.

Vitellin, the corresponding lipid-free protein, was more difficult to deal with since it is practically insoluble in water or dilute salt solutions. However, with the aid of a little very dilute NaOH and in other cases with saturated urea solution, the vitellin was converted into a fine cream for injection. Unfortunately, this suspension proved very toxic to the two rabbits used for this experiment, and they both died in just under 1 min. after the injection. By withdrawing blood from the heart and removing selected organs at once, material was obtained for a study of the distribution of the injected antigen. The results (Table 3) showed that a considerable amount, possibly as much as half of the injected antigen, had left the blood stream in the first minute after the injection, most of the missing antigen being

found in the liver and lungs. Calculations have shown that the radio-P in the blood present in the lung and liver samples analyzed could account for a small fraction only of the radio-P content of these organs.

DISCUSSION

The site of antibody formation in the animal body is not clearly established, but it is believed by many investigators that antibodies, which, according to the most widely accepted view, are essentially modified plasma globulins, are synthesized in the same place as are the normal plasma proteins. Madden & Whipple (1940), from the evidence of their own and other investigations, concluded that the liver takes an important part in the manufacture of the normal plasma proteins, and it is not unreasonable to believe that this organ is partly or wholly responsible for the synthesis of antibodies against foreign proteins and other antigens. There is also evidence that the reticulo-endothelial system as a whole takes part in this synthesis (cf. reviews by Landsteiner, 1945, pp. 146-7; Boyd, 1943, pp. 62-4).

Whatever the site of antibody formation, it would seem almost certain that the injected antigen, or some fairly large fraction of the molecule, will be located in these tissues at some time after its injection. Working on this hypothesis several investigators have examined various tissues at intervals after the injection of readily detectable antigens. Haurowitz & Breinl (1932), for example, used an arsenic-containing azoprotein antigen and found that most of the arsenic remained in the liver and 'marrow bones'. Other evidence of the localization of much of the injected antigen in the liver was obtained by Rous & Beard (1934) using ferromagnetic iron oxide and by Sabin (1939) with the aid of a coloured azoprotein.

In our experiments we have used ³²P-containing protein antigens, although these are not ideal labelled

Table 3. *Radio-P content of the blood and some organs of rabbits shortly after the intravenous injection of ³²P-containing vitellin*

(Rabbits nos. 634 and 635, each of 2.7 kg., received 5.0 and 4.0 ml. respectively of a solution or suspension of radio-vitellin; the amount of radio-P injected was 2575 and 2060 μ g. respectively. Both rabbits died about 1 min. after the injection. Blood was then immediately taken from the heart, and the organs were excised as quickly as possible and treated as described in the text.)

	Radio-P content			
	Rabbit no. 634		Rabbit no. 635	
	(μ g.)	As % of radio-P injected	(μ g.)	As % of radio-P injected
Blood	2025*	79*	1080*	52*
Kidneys	37	1.4	<10	<0.5
Lungs	481	19	339	16
Spleen	0	0	0	0
Heart	10	0.4	0	0
Liver	402	16	354	17

* Assuming a blood volume of 50 ml./kg. of body wt., and uniform distribution of the radio-P throughout the blood.

proteins for such an investigation. The phosphorylated proteins, like the azoproteins, are artificial complexes, and most of the linkages between the introduced phosphate groups and the protein molecule are fairly readily split at 37° (Heidelberger, Davis & Treffers, 1941; Bournsnel *et al.* 1948); this, together with the fact that the liberated inorganic phosphate will undoubtedly take part in metabolic reactions unrelated to antibody formation, renders results obtained with phosphorylated proteins of limited value. The localization of ^{32}P in any tissue after the injection of these proteins should only be regarded as being immunologically significant if it occurs fairly soon after the injection, though some of the phosphate groups may remain attached to the protein for some considerable time after the injection. The use of vitellin and lipovitellin is not open to the same objections, since these are natural proteins containing firmly bound phosphate. On the other hand, they are insoluble or almost insoluble in water and dilute salt solutions, though this is not an insuperable difficulty in experiments involving the tracing of injected antigen. A much better antigen for these investigations is the $\beta\beta'$ -dichlorodiethylsulphone-protein compound (Berenblum & Wormald, 1939; Bournsnel, Francis & Wormald, 1946b), and now that supplies of ^{35}S have again become available in this country our investigations with this antigen are being continued.

The results of the experiments described here have shown that the injected antigens are rapidly removed from the circulating blood, and the two experiments with injected vitellin (Table 3) show that much of the antigen is removed by the liver during the first minute or so after the injection. (The presence of a considerable amount of ^{32}P in the lungs in this experiment is not regarded as necessarily significant, for it seems probable that the vitellin particles injected, or those precipitated when the suspension was injected into the vein, were unable to pass through the lung capillaries.) This removal of injected foreign particles by the liver is similar to that observed by Jones, Wrobel & Lyons (1944) and Dobson, Kelly, Jones & Gofman (1947), in their experiments with ^{32}P -containing chromic phosphate; this injected compound became localized specifically in the liver and spleen, and practically all was removed in a single passage of the blood through the liver. Similar results were obtained with other radioactive inorganic colloids (Dobson *et al.* 1947), but in our experiments the injected protein particles were not taken up by the spleen during the first minute or so after the injection. It is possible that the spleen may take up injected antigens or other colloids at a later stage, but the results obtained by Sheppard, Wells, Hahn & Goodell (1947) suggest that there may be fundamental differences in the phagocytic removal of different injected colloids. Using radio-

active isotopes these authors found, for example, that the injection of colloidal gold into men and dogs resulted in a high concentration of this element in both the liver and spleen, whereas the injection of colloidal manganese dioxide led to a high concentration of manganese in the liver but not in the spleen.

Specific deposition of radioactive colloid in the liver, and the resulting specific irradiation of that organ, led to a marked depression of deoxyribose nucleic acid metabolism (Jones, Kelly & Lawrence, 1947). One of the objects of our own investigations with ^{32}P -containing lipovitellin and vitellin was to obtain specific irradiation of the reticulo-endothelial system and any other tissue in which the injected antigen is localized. The relatively short half-life period of ^{32}P (14.3 days) and the probability that the phosphoproteins will be slowly hydrolyzed in the tissues may render these egg-yolk proteins specially suitable for this type of investigation. For some of these experiments we propose to use ^{32}P -containing bacterial nucleoproteins, for preliminary experiments made in collaboration with Prof. L. P. Garrod have shown that appreciable amounts of ^{32}P can be introduced into protein fractions of streptococci and coliform organisms.

SUMMARY

1. ^{32}P -containing proteins have been used to study the fate of intravenously injected antigens. The antigens used were lipovitellin and vitellin obtained from eggs laid by hens injected with ^{32}P -containing inorganic phosphate, and artificially phosphorylated human serum proteins.

2. The injected proteins quickly disappeared from the blood stream. Less than one quarter of the injected phosphorylated human serum proteins was detectable in the blood 3 hr. after the injection, and the disappearance of vitellin and lipovitellin was even more rapid, since only about one eighth of the injected protein could be detected in the blood 5 min. after the injection.

3. Appreciable amounts of ^{32}P were found in the liver and lungs of two rabbits 1 min. after the injection of a suspension of vitellin. The presence of this material in the lungs was possibly due to the retention, by the lung capillaries, of vitellin which was precipitated when the material was introduced into the blood stream.

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***p*-Ethylphenylsulphuric Acid in Goat Urine**

By J. K. GRANT, *Department of Biochemistry, University of Edinburgh*

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Although it was suggested by Baumann in 1879 that *p*-ethylphenol might be formed in the animal body by the degradation of tyrosine, the presence of this substance was not reported until 1927, when Walbaum & Rosenthal (1927) and Pfau (1927), isolated it from the dried scent glands of the beaver. More recently, Lederer (1943, 1946) isolated *p*-ethylphenol from an extract of acid-hydrolyzed pregnant mare urine.

In the present work the isolation of *p*-ethylphenylsulphuric acid from urine as the potassium salt is reported for the first time. This substance was initially obtained from the urine of an ovariectomized goat, which had received large doses of progesterone and hexoestrol. Subsequently it was also isolated from the urine of a normal goat.

In view of the belief (Williams, 1947) that *p*-cresol is quantitatively the most important phenol in the urine of vertebrates, it is noteworthy that no clear-cut evidence has been obtained for the presence of *p*-cresyl sulphuric acid in the urine examined. The fact that the derivatives of *p*-ethylphenol, prepared from the hydrolysis product of the sulphate, required frequent recrystallization before constant melting points could be obtained, might suggest that the isolated substance was contaminated with

appreciable amounts of the sulphate of *p*-cresol or of other phenols. Nevertheless, the present work indicates that in the goat *p*-ethylphenylsulphuric acid is excreted in larger amount than *p*-cresylsulphuric acid.

EXPERIMENTAL

The experimental animals were mature ovariectomized goats on a diet of hay and a mixture of bran, linseed cake, oats and beans. For the purpose of lactation studies under the direction of Dr S. J. Folley at the National Institute for Research in Dairying, they received 1 mg. hexoestrol and 40 mg. progesterone daily. Dr Folley and Mr A. T. Cowie kindly arranged for the collection of urine, and for its dispatch to this laboratory.

The urine was collected quantitatively under butanol. Daily collections were stored in the refrigerator. The pooled 7-day collections received were worked up for conjugated substances. Normal goat urine was collected similarly from an animal receiving no treatment.

Extraction of urine

Urine (3.5 l.) from a treated goat was acidified to pH 3 with HCl, and rapidly extracted with butanol at low temperatures. The butanol extract was washed with $\frac{1}{3}$ -NaOH, and water, and evaporated to dryness under reduced pressure. The residue was dissolved in water, chilled to 0°,