IN VITRO PRODUCTION OF DIPHTHERIA ANTITOXIN BY TISSUES OF IMMUNIZED ANIMALS. III. INCORPORATION OF AMINO ACIDS INTO ANTIBODY; RELATIONSHIP TO ANTIBODY SYNTHESIS AND SENSITIVITY RELATIVE TO OTHER METHODS

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Received for publication July 28, 1958

In previous studies from this laboratory (Stavitsky, 1955*a*; Stavitsky and Wolf, 1958; Wolf and Stavitsky, 1958) evidence was presented for the *de novo* synthesis of diphtheria antitoxin when lymphoid tissues from immunized rabbits were incubated *in vitro* in a suitable medium. Further data obtained both *in vitro* (Stavitsky and Wolf, 1958; Wolf and Stavitsky, 1958) and *in vivo* (Taliaferro and Talmage, 1955; Stavitsky, 1955*b*, 1957*a*, 1958*a*) systems indicated that antibody synthesis required amino acids rather than more complex polypeptide or protein precursors. A synthetic medium was then developed which supported appreciable antibody synthesis as well as incorporation of radioactive amino acids into antibody by immunized lymphoid tissue *in vitro* (Wolf and Stavitsky, 1958). However, before the incorporation technique could be utilized for the investigation of aspects of antibody synthesis it was essential to ascertain whether incorporation of amino acids represented genuine synthesis of antibody. There has been much discussion (reviewed by Tarver, 1954) whether incorporation is tantamount to synthesis or whether incorporation represents exchange of protein amino acids rather than synthesis. The present study was designed primarily to determine the relationship between incorporation and antibody synthesis. Once a reasonable correlation between these phenomena was established a second purpose was to determine how sensitive a criterion of the antibody-producing capacity of a tissue was provided by the isotopic technique relative to other techniques. Finally after establishing the great sensitivity of the isotopic technique, it was applied to the preliminary study of a number of immunological phenomena which have hitherto been rather difficult to analyse.

MATERIALS AND METHODS

Animals.—The donor rabbits were male albinos which weighed 2·5–3 kg. The recipient rabbits usually also were male albinos which weighed 0·5–1 kg. They were fed Purina rabbit checkers and given water *ad libitum*.

Antigens.—The " Purogenated " alum-precipitated and fluid diphtheria toxoid and bovine γ-globulin preparations were described in a previous paper (Stavitsky, 1957*b*). The author is grateful to Lederle Laboratories for furnishing the diphtheria toxoid. In the isotopic experiments a purified diphtheria toxoid (K 39/50 kindly furnished by Dr. Inga Scheibel) was employed, which contained 2650 L_f units per mg. protein N. Ovalbumin (thrice recrystallized—Armour Laboratories) was used for the production of rabbit antisera.

* This investigation was conducted during the tenure of an Established Investigatorship from the American Heart Association.

Crystalline bovine serum albumin was purchased from Armour Laboratories. The rabbit γ-globulin was derived from an anti-pneumococcus Type I rabbit serum, generously provided by Dr. E. L. Smith of the University of Utah. The preparation and properties of this globulin were recently described (McFadden and Smith, 1955). The preparation contained 92 per cent of γ component with an S value of 6·3 and 8 per cent of a component with a value of 8·3.

Antisera.—The ovalbumin antiserum was prepared by the intravenous injection of alum-precipitated ovalbumin into rabbits as described by Vaughan and Kabat (1953). This procedure yielded consistently potent antisera containing 0·5–7·0 mg. specific antibody/ml. of serum. The bovine γ-globulin antiserum was prepared by the intravenous injection of alum-precipitated bovine γ-globulin by the same procedure as described above for ovalbumin. Rabbit antisera to diphtheria toxoid were prepared by 2 or more intravenous and foot pad injections of 16–32 L_f units alum-precipitated toxoid separated by an interval of at least 28 days and bleeding 7–8 days after the second injection. Chicken anti-rabbit γ-globulin sera were produced by the intravenous inoculation of 3·0 mg. of purified γ-globulin 4 times a week for 3 weeks followed by a bleeding 7 days after the last injection. Four antisera were prepared and tested by the quantitative precipitin procedure (Kabat and Mayer, 1948). One of these showed a single precipitin peak, clear-cut antibody excess, equivalence and antigen excess zones. Only this antiserum was used in the present study.

Antibody assay.—Antibody was measured by the haemagglutination procedure. " Purogenated " fluid diphtheria toxoid was adsorbed on sheep erythrocytes which had been treated with tannic acid according to a modification (Stavitsky, 1954*a*) of the Boyden procedure (Boyden, 1951) and the cell-toxoid complex was agglutinated by antitoxin. The potency of the antiserum was designated as the reciprocal of the highest dilution which yielded a ++ agglutination pattern (Stavitsky, 1954*a*). All the materials for serological assay from a single experiment were titrated on the same day because of the variability in titres of the same antiserum against different batches of protein-conjugated cells. All of the media employed in this study were found to be without effect upon the haemagglutination assay when a standard antiserum was mixed with the materials (1/10) and the titres of these mixtures compared with that of the same antiserum mixed similarly with saline.

In early experiments the amount of antitoxin produced *in vitro* was calculated as follows : From the sum of the reciprocal of the tissue and medium titres was subtracted the base line titre of the extract of unincubated tissue. The validity of this figure depended on the reproducibility of the weights of the tissue samples employed for homogenization and incubation. The average net weight of the lymph node fragments of a large number of samples was 14 mg. with a range of 7 to 21 mg., the average of the spleen fragments was 12 mg. with a range 5 to 20 mg. Therefore, unless the net antibody production (tissue plus medium titres minus base line titre) was at least 5 times the base line titre the experiment was disregarded.

In most of the experiments reported here the data were recalculated on the basis of 10 mg. of tissue and the net antibody production expressed as haemagglutinating units of antitoxin produced per 10 mg. of tissue. Since previously (Stavitsky, 1954*b*) a relationship had been found between haemagglutination titre and antitoxin unitage derived from biological assay, the haemagglutinating units could be expressed at least approximately in terms of units of antitoxin. Moreover, since data are available on the amount of protein per unit of antitoxin (Cohn and Pappenheimer, 1949) the titre may be expressed also in terms of quantities of antitoxic protein.

The *ring reaction* was performed in 6 × 50 mm. tubes. The antiserum was placed in the bottom of the tube and antigen carefully layered on top of the serum. The technique of Heidelberger (Kabat and Mayer, 1948) was employed with slight modification for quantitative precipitin analysis. The antigen-antibody precipitates were washed twice in ice-cold saline and the protein analysed by the Lowry method (Lowry, Rosebrough, Lewis and Randall, 1951).

Immunization and tissue culture.—Rabbits had 60 μg. (20 L_f units) of alum-precipitated diphtheria toxoid injected. Injections were made intravenously when spleens were to be cultured or into the hind foot pads when the popliteal lymph nodes were employed. When the mesenteric lymph nodes were to be utilized 4 L_f of antigen sometimes was injected directly into these nodes. Unless indicated otherwise injections were made on days 1 and 30 and organs removed for culture on day 33 since a 3-day interval between booster injections was shown to be optimal (Stavitsky and Wolf, 1958). The tissues were cut into fragments about 3 mm^3. All procedures were done aseptically. About 20 mg. of fragments (3 pieces) were placed in a 13 × 100 mm. tube containing the medium. The tubes were stoppered, placed

in a roller machine (12 rotations/hr.) and incubated for 20–24 hr. After incubation the medium was decanted and clarified by centrifugation. Since 75–90 per cent of the antibody was in the medium after incubation (Stavitsky, 1955*a*), the antibody content of the tissue at this time usually was not measured. For analysis of the tissue before incubation it was homogenized in cold physiological saline since this method was shown (Stavitsky, 1955*a*) to yield maximal amounts of antibody.

Media.—Fischer's medium (Fischer, Astrup, Ehrensvaard and Oehlenschlager, 1948), W medium (Stavitsky and Wolf, 1958), 79, 88 and 112 media (Wolf and Stavitsky, 1958) and Earle's saline (Earle, 1948) have been described previously.

Homotransplantation techniques.—Lymphoid cells were isolated by teasing the spleen or lymph nodes with the prongs at the cut edge of 60-mesh stainless steel wire mesh. One set of prongs was employed to anchor the tissue and the other to comb out the cells. The cells were suspended in Earle's saline containing 0·125 per cent gelatin. All cell transplants were made intraperitoneally. A small portion of each cell suspension was saved for analysis and the larger part transplanted. The recipient animals were untreated. Although the recipients were not treated to abolish their capacity to produce antibody against antigen in the donor cells, the antibody produced in them after transplantation appeared too rapidly (4–8 days) to be due to active immunization (Stavitsky, unpublished).

Isotope procedures.—^{35}S-L-methionine (16·3 μC/mg.) and ^{35}S yeast protein hydrolysate (about 0·58 μC/ml.) were obtained from Abbott Laboratories or Schwarz Laboratories. The hydrolysate contained radioactivity in the following precentages of labelled compounds as determined by paper chromatography : 30–50 per cent L-cystine, 20–40 per cent L-methionine and 5–15 per cent glutathione. In occasional experiments, ^{14}C DL-histidine (labelled in the 2-ring position), and ^{14}C L-leucine (1 C labelled) were employed with essentially similar results as regards specific and non-specific precipitation with the antigen-antibody aggregates.

For the determination of radioactivity of antibody the medium was centrifuged at the end of the incubation and radioactive substances, including labelled proteins which might be adsorbed non-specifically on the antigen-antibody aggregate and give a falsely high value, were removed by a heterologous antigen-antibody precipitate, as described by Keston and Katchen (1956). One to 2 mg. of egg albumin-anti-egg albumin or bovine γ-globulin-anti-bovine γ-globulin precipitates at equivalence point were employed per tube for this purpose. It was found that the formation of neither of these precipitates removed diphtheria antitoxin from the medium. Diphtheria antitoxin was precipitated by a concentration of K 39/50 toxoid which precipitated 96–99 per cent of the antibody as determined by haemagglutination titration of the supernatant. From 1–4 per cent of the antitoxin always was demonstrable in the supernatant, presumably due to agglutination of toxoid-conjugated cells by soluble antigen-antibody complexes. All of the precipitates were washed twice in cold saline containing 250 μg. of L-methionine and L-cystine per ml. and dialyzed in the cold for 2 days against 3 changes of 3 litres of saline. Visking 8/32 tubing had to be used for the dialyses because tubing of wider diameter apparently did not possess adequate surface area and dialysis in the larger tubing did not result in the attainment of constant radioactivity in 2 days. The precipitates were collected by centrifugation and resuspended in saline and dissolved in 0·25 ml. of 0·1 N NaOH. 100–750 μg. of protein were pipetted into a steel planchet, a drop of 1 per cent Aerosol OT (Eimer and Abend, New York) added, and the precipitate dried under an infra-red lamp. The radioactivity was determined with a nuclear flow counter, occasionally with a Tracerlab Autoscaler. The amount of antibody in the precipitate was calculated from the equivalence ratio of antibody to antigen for each antigen-antibody system so that the radioactivity of the immune precipitate could be recalculated in terms of the activity of the antibody itself. The observed specific radioactivity of the homologous precipitate (toxoid-antitoxin or other antigen-antibody precipitate) minus the observed radoactivity of the heterologous precipitate yielded the actual specific radioactivity of the homologous precipitate from which the specific radioactivity of the antitoxin or other antibody could be calculated.

As a check on the ability of these methods to eliminate non-specific radioactivity, it was found that toxoid-antitoxin precipitates prepared after incubation of tissues from non-immunized animals in a radioactive medium contained very little radioactivity (background or 1/3 greater).

Similar amounts of antigen-antibody precipitate were obtained when antigen and antibody were added to the various media employed in this study. It was assumed, therefore, that the medium employed did not affect the recovery of antibody.

The early isotope experiments were carried exactly like the synthesis ones with approxi-

mately 20 mg. of tissue being incubated in 1 ml. of medium with rotation in a roller assembly. The media from 3 tubes were combined for each determination. Later, however, 9 fragments (approximately 60 mg. of tissue) were incubated in 3 ml. of medium in a 20 ml. beaker in a Dubnoff metabolic shaker with gentle agitation. No striking difference in results was observed with these two methods of incubation and gentle movement of the tissues.

EXPERIMENTAL

Correlation of results of in vitro *and homotransplantation studies*

In previous studies (Stavitsky, 1955*a*) the sensitivity of the *in vitro* and homotransplantation procedures was compared by using small fragments of tissues from immunized animals for *in vitro* culture and teasing apart the larger part to provide cells for transplantation. The haemagglutination assay (Boyden, 1951; Stavitsky, 1954*a*) was used in both procedures. There was only a fair correlation between the results obtained by these 2 techniques. Occasionally the tissue produced no antibody *in vitro*, whereas isolated cells from this tissue synthesized a considerable amount of antibody upon homotransplantation. Other data in the same study (Stavitsky, 1955*a*) indicated that by both methods the antibody-producing capacity of the lymph node was maximal on the third day after antigenic restimulation of the rabbit.

The purpose of the present experiments was to obtain more definitive data regarding the relative sensitivity of the *in vitro* and homotransplantation procedures for the determination of the antibody-producing capacity of tissues in order to relate the *in vitro* activity of the tissues to an *in vivo* process. Although previous data (Roberts and Dixon, 1955; Taliaferro and Talmage, 1955; Stavitsky, 1955*a*, 1957*a* and 1958*a*; Stavitsky and Wolf, 1958) had indicated that both the *in vitro* and transplantation techniques were measuring essentially the same process of *de novo* antibody synthesis from amino acids, it was felt that more clear-cut information on this point was essential before embarking upon a study of the process of antibody formation entirely with an *in vitro* system.

Table I presents the results of a number of typical experiments. Occasionally, as noted previously (Stavitsky, 1955*a*), the homotransplantation appeared more sensitive than the *in vitro* procedure (Expts. 6, 7). But often the *in vitro* procedure appeared more sensitive (Expts. 1, 4, 8).

The formation of antibody upon transplantation of lung cells (Expt. 6) is interesting because this organ was not found active in a previous study (Stavitsky, unpublished) in which diphtheria toxoid was injected only twice. In the present experiment the toxoid was injected repeatedly. This finding confirms the results of recent experiments with egg albumin (Askonas and Humphrey, 1958) in which the lung was found to be extremely actively engaged in antibody synthesis in animals which had received many injections of antigen. The inactivity of the liver (Expt. 5) corresponds to the results of previous studies (Stavitsky, 1957*a*).

Correlation of results of in vitro *synthesis and isotopic studies*

The previous experiments had indicated that the *in vitro* synthetic procedure was at least as sensitive and often a more sensitive criterion of the antibody-producing ability of tissues than the *in vivo* homotransplantation procedure. With this information and the data (Stavitsky, 1955*a*, 1957*a* and 1958*a*; Stavitsky and Wolf, 1958) which suggested strongly that *de novo* synthesis of antibody

TABLE I.—*Relative Sensitivity of* In vitro *Culture and Homotransplantation Methods*

Expt. No.	Preparation of animal	Tissue used	Results—reciprocal titre		
			Culture	Transplant	
				Peak	Days
1	32 Lf APT 40 mg. AP-BGG intraven. days 1, 40 and 58 ; tissue removed on day 61	MES	80 (Tox) 160 (BGG)	0 (Tox) 0 (BGG)	— —
2	40 mg. AP-BGG intraven. days 1 and 64 ; tissue removed on day 67	Spleen LLN MES	560 140 80	80 Spleens from rabbits in Expt. 2 and 3	2
3	40 mg. AP-BGG intraven. days 1 and 64 ; tissue removed day 67	Spleen LLN MES	1280 — —	160 LLN + MES from rabbits in Expt. 2 and 3	2
4	40 mg. AP-BGG intraven., 2·5 mg. each foot pad days 1 and 30 ; tissues removed day 33	Spleen LLN RLN MES	40 40 40 40	0 0 0 0	— — — —
5	32 Lf APT IV and 8 Lf APT each foot pad days 1, 5, 9, 14, 23, 26 ; tissues removed day 29	Liver Spleen LLN	— 20 20	— 80 80	— 3 3
6	Same as 5	Spleen Lung LLN MES	0 0 0 0	0 40 0 —	— 6 — —
7	40 mg. AP-BGG intraven., 2·5 mg. each foot pad day 1 ; 20 mg. BGG intraven. and 10 mg. each foot pad day 13 ; tissues removed day 16	Spleen LLN RLN MES	0 0 0 0	80 0 0 0	7 — — —
8	100 mg. BSA intraven. day 1 and 37 ; tissues removed day 40	Spleen MES	120 0	0 0	— —
9	5·6 mg. AP-EA intraven., 1·4 mg. AP-EA foot pads day 1 ; 1·4 mg. AP-EA intraven. ; 0·7 mg. foot pads day 38 ; tissues removed day 41	Spleen LLN MES	40 20 10	40 0 0	4 — —

APT = alum-precipitated diphtheria toxoid ; AP-BGG = alum-precipitated bovine γ-globulin ; AP-EA = alum-precipitated egg albumin ; LLN = left popliteal lymph node ; RLN = right popliteal lymph node ; MES = mesenteric lymph node.

from free amino acids was occurring *in vitro* when tissues of immunized rabbits were cultivated in a suitable medium, attention was focused upon the relationship of *in vitro* incorporation of amino acids into antibody to the actual synthesis of antibody. In a previous study (Wolf and Stavitsky, 1958) a relationship between these phenomena was suggested by the findings that the optimal concentrations of certain amino acids in the medium were required both for maximal *in vitro* incorporation as well as synthesis of antibody, and that the same W medium (Stavitsky and Wolf, 1958) supported maximal antibody synthesis as well as maximal incorporation.

Table II presents the results of a number of experiments in which the synthetic and incorporation processes were studied in parallel with the same tissue preparation and *in vitro* systems. It is clear that there was some correlation between the

2 types of data. In general a tissue which was actively synthesizing antibody, as evidenced by the haemagglutination titre of the culture medium, was also actively engaged in the incorporation of radioactive amino acids into antibody (Expts. 10, 12, 13). With increased time of incubation the extent of both processes tended to increase (Expts. 10 and 14). Usually tissues low in synthetic ability also tended to be low in incorporating capacity (Expt. 14) and a tissue lacking synthetic ability also lacked incorporating capacity (Expt. 11). However, whereas a preparation which produced antibody also incorporated amino acid into antibody, on occasion preparations, which did not seem to be synthesizing antibody according to the haemagglutination assay, were nevertheless found to be incorporating radioactive amino acid into antibody (Expts. 10 at 24 hr; 14 and 15). A discrepancy between the 2 assays was found in Expt. 13 in which there was no obvious correlation between the haemagglutination titres and the specific radioactivity of the antibody in the immune precipitates prepared from these media. This discrepancy will be discussed later.

TABLE II.—*Relative Sensitivity of* In Vitro *Culture and Isotopic Methods*

Expt. No.	Preparation of animal	Tissue	Medium	Isotope (μc. M/tube)	Hr. of incub.	Results: Culture titre	Results: Incorporation—c/m/mg. antibody: Specific	Results: Incorporation—c/m/mg. antibody: Non-specific
10	APT foot pads	LLN	88	2·5	24	160	326	147
					48	160	503	30
		RLN	88	2·5	24	0	400	186
					48	240	838	161
		Spleen	88	2·5	48	0	1,100	183
11	Ditto	LLN	W	2·5	24	0	349	349
		RLN	W	2·5	24	0	434	517
12	APT intraven.	Spleen	88	2·5	24	320	465	82
			ES			40	98	72
13	APT foot pads	LLN	W	2·5	24	80	10,800	900
			FS	—	—	93	3,100	700
			79	—	—	106	2,160	490
			88	—	—	93	1,100	860
			112	—	—	26	2,410	660
14	Ditto	RLN	W	2	20	0	106	127
					48	0	280	112
		LLN	W	2	20	0	100	104
					48	0	201	131
15	AP-EA intraven.	POP	W	0·7	24	0	12,000	32,700
		MES	—	—	—	0	14,000	24,600
		Spleen	—	—	—	0	11,200	24,300

M = ^{35}S L-methionine; H = ^{35}S yeast protein hydrolysate; foot pads = hind pads; intraven. = intravenously; ES = Earle's Saline; FS = 20 per cent rabbit serum + 80 per cent Fischer's medium.

Table II also presents evidence of the specificity of the isotopic assay. In every experiment the medium was treated first with a heterologous antigen-antibody system before the homologous antigen-antibody precipitate was prepared. It is clear from the data that the antibody in the homologous antigen-antibody precipitate contained many times as much specific radioactivity as that in the

heterologous antigen-antibody precipitate. In 2 experiments (12–24 hr. in ES, and 14–20 hr.) since the specific antibody did not contain more radioactivity than the heterologous antibody it was concluded that specific incorporation had not occurred.

Although the foregoing data suggested some correlation between the processes of incorporation of amino acids into antibody and antibody synthesis, this was far from absolute. Therefore, the relationship between these processes was analysed further by determining simultaneously the effect of various inhibitors on both processes. Table III indicates that there was a roughly corresponding decrease in incorporation and antibody synthesis when various inhibitiors were employed. Again, as noted in Table II, the relatively greater sensitivity of the isotopic procedure permitted the detection of apparent antibody-forming capacity in certain tissues in the absence of haemagglutinating antibody (Expts. 18 and 19). The inhibition of methionine incorporation by an analog of phenylalanine (Expt. 16) indicated that more than one amino acid was utilized for the synthesis of antibody. The requirement for energy for antibody synthesis and incorporation was shown by the inhibition of both processes under anaerobic conditions (Expt. 20). Arsenate and potassium cyanide also inhibited both processes, but inhibition was less consistently observed than with helium. The requirement for intact cells for both processes was demonstrated by inhibition of synthesis and incorporation when the tissues were homogenized or heated before incubation (Expts. 19 and 20). The results of comparing both synthesis and incorporation by isolated cells rather than fragments (Expt. 21) suggested that either a number of cells

TABLE III.—*Effect of Various Physico-chemical Factors on Antibody Synthesis and Incorporation*

Expt. No.	Tissue	Hr. of incub.	Isotope	Other factors	Results: Titre	Results: c/m/mg. antibody
16	POP	48	2·5 μc. M/tube	88	320	38,000
				ES	40	3,000
				88–pFlφal	40	9,400
17	Spleen	48	2·6 μc. M/tube	W	20	80,000
				W-F + T6 ×	0	0
18	,,	6	1 μc. H per beaker	W–37°	0	86,000
				W–5°	0	13,900
19	,,	1	2 μc. H per beaker	W	0	256
				W–Homog.	0	0
				W–60°/45′	0	50
20	POP	3	2 μc. H per beaker	W	120	7,470
				W–Helium	40	1,590
				W–Homog.	40	2,170
				W–pFlφal	80	4,000
21	Spleen	3	2 μc. H per beaker	Fragments (55 mg)	20	9,230
				Cells (400 mg.)	20	10,200
	POP	3	2 μc. H per beaker	Fragments (28 mg.)	160	14,760
				Cells (150 mg.)	—	24,300

POP = popliteal lymph node; M = ^{35}S methionine; H = ^{35}S yeast protein hydrolysate; pFlφal = 1 mg. of analogue per 1 ml. of medium; F + T 6 × = frozen and thawed 6 times; Homog. = tissue homogenized, whole homogenate incubated with isotope in medium; 60°/45′ = tissue heated at 60°/45′ before incubation; fragments (55 mg.) = 55 mg. of fragments incubated in medium; cells (400 mg.) = the cells isolated from 400 mg. of tissue were incubated.

or synthetic ability was destroyed during the process of isolation of the cells because the cells from large amounts of tissue were required to observe antibody synthesis or incorporation comparable to that obtained with smaller amounts of tissue fragments.

Further evidence of the relationship of incorporation to actual antibody synthesis was provided by the results of experiments in which a limited amount of radioactive histidine was placed in the medium. Under these conditions (Table IV) antibody of progressively decreasing specific activity was synthesized, while the total amount of radioactive antibody was increasing. The decrease in specific activity was also reflected in the decrease in the ratio of specific to non-specific radioactivity with time. Presumably, the limited amount of radioactive amino acid in the medium was diluted with endogenous non-labelled amino acid or with non-labelled amino acids liberated by the breakdown of tissue protein, resulting in progressive dilution of the radioactivity of the amino acid precursor in the medium. Such a relationship between incorporation and synthesis would be expected if the amino acid were being utilized for protein synthesis, but would be less likely if the amino acid were being exchanged. These data provide further evidence of the superior sensitivity of the isotopic technique since specific incorporation was observable after incubation of tissue for 1 hr. whereas a haemagglutination titre was not obtained until the tissues had been incubated for 6 hr.

TABLE IV.—*Specific Activity of Antibody and Total Radioactivity of Antibody Synthesized from Limiting Concentrations of ^{14}C-histidine**

Tissue	Time of incub. (hr.)	Results			
		HA titre	c/m/mg. Ab	Q†	Total activity (Titre × Sp. Act.)
Left popliteal node	1	0	3,920	30	39,200
	3	0	2,080	19·2	40,160
	6	40	7,350	24·7	29,400
	24	80	4,900	12·9	39,200
Right popliteal node	1	0	2,940	26·8	26,800
	3	0	2,300	12·6	23,000
	6	20	4,760	12·5	95,200
	24	80	2,980	6·92	238,400

* DL-histidine 0·5 μc. (2-ring ^{14}C).
† Q = ratio of specific to non-specific radioactivity.

Evidence that co-precipitated material is γ-globulin

In those experiments in which haemagglutinating antibody was not detected in the medium it appeared desirable to establish with greater certainty that the co-precipitated material was antibody γ-globulin. It was found that dialysis of the medium and precipitation of the γ-globulin from the medium by the method of Eisen and Pressman (1950) resulted in concentration of the co-precipitable material in the globulin rather than albumin fraction. Pre-treatment of the medium with rabbit γ-globulin- chicken anti-rabbit γ-globulin precipitates resulted in considerable diminution of the amount of radioactivity subsequently precipitated by the specific antigen-antibody system and thus provided further evidence that the co-precipitated material was γ-globulin.

Observations of various aspects on antibody synthesis by isotopic method

The preceding data indicated that the isotopic method provided a very sensitive criterion of the occurrence of antibody synthesis. This method was, therefore, applied to the preliminary re-examination of a number of aspects of the problem of antibody synthesis as well as to the exploration of new aspects of the problem. Table V provides further evidence of the superior sensitivity of the isotopic method by comparison with other methods. An apparent genetic block in the synthesis of a distinct species of antibody (anti-egg albumin) by a rabbit, as evidenced by a lack of demonstrable circulating antibody, was accompanied by an inability of its tissues to produce either haemagglutinating antibody or to incorporate amino acids into antibody (Expt. 22). Whereas it was previously believed (Stavitsky, 1955*a*) that the injection of diphtheria toxoid into the left rear foot pad did not stimulate antibody synthesis by the right popliteal lymph node, the incorporation technique indicated that the right node was active (Expt. 23). The activity of the spleen following injection of antigen into the foot pads (Stavitsky, 1955*a*, 1957*a* and 1957*b*) was confirmed by this method (Expt. 24).

TABLE V.—*Application of Isotopic Procedure to Study of a Variety of Immunological Phenomena*

Expt. No.	Preparation of animal	Tissue	Isotope	Other factors	Hr. of incub.	Results		
						Titre	c/m/mg. antibody	
							Specific	Non-specific
22	4 weekly courses of AP-EA intraven.: tissues out 7 days after last injection§	POP	0·7 μc. M/tube	—	48	0	12,000	32,700*
		MES	—	—	—	0	14,400	24,600
		Spleen	—	—	—	0	11,200	24,300
23	APT left foot pad on days 1 and 30; RLN out day 33	RLN	2·5 μc. H/tube	—	24	10	11,390	2,280†
24	APT foot pads days 1 and 30; tissues out day 33	Spleen	2 μc. H/beaker	—	48	0	1,100	183†
		LLN	—	—	—	160	500	30
		RLN	—	—	—	180	838	161
25	APT foot pads day; tissue out day 365	LLN	2·5 μc. H/beaker	W	3	—	104,200	—
				W 60°/45′	—	—	29,200	—
26	APT 32 Lf each foot pad day 1; 4 Lf left pad, 40 Lf right pad day 300; tissues out on day 303	LLN	2 μc. H/beaker	—	1	0	3,920	130‡
		RLN	—	—	1	0	2,940	110
27	APT intraven. and foot pads days 1, 42, 50; tissues out day 58	Spleen	2 μc. H/beaker	—	24	0	6,900	—
		POP	—	—	—	0	10,100	—
		MES	—	—	—	0	8,650	—

M = ^{35}S L-methionine; H = ^{35}S yeast protein hydrolysate; 60°/45′ = tissues heated at 60°/45 min. before incubation in medium.

§ No antibody found by ring reaction or quantitative precipitin reaction.

* Toxoid-antitoxin precipitate used as heterologous system.

† EA-Anti-EA used as heterologous system.

‡ BGG-Anti-BGG used as heterologous system.

Antibody synthesis by the mesenteric lymph nodes, which were difficult to stimulate except by injections of antigen directly into this organ or by the intravenous inoculation of large amounts of soluble antigen (Stavitsky, 1957*a* and 1957*b*), was demonstrated readily by the isotopic method employing the tissues of animals which had received alum-precipitated antigen intravenously and into the foot pads (Expt. 27). Employing the isotopic technique it was possible to demonstrate antibody synthesis by a variety of organs 8 days after the booster injection when haemagglutinating antibody was not demonstrable.

DISCUSSION

Although there is good evidence that the incorporation of radioactive amino acids into antibody by the intact immunized animal represents antibody synthesis (Taliaferro and Taliaferro, 1957), there appeared to be a need for more definitive evidence regarding the identity of these 2 processes as studied *in vitro*. Keston and Katchen (1956) showed that there was considerable non-specific adsorption of radioactivity in *in vitro* antibody-forming systems. The finding that the synthesis of antibody *in vitro* represented *de novo* synthesis of this protein probably identical to the process in the intact animal (see Stavitsky, 1957*a* and Stavitsky and Wolf, 1958) presented the possibility for this study of the relationship of incorporation to synthesis.

The present data indicate that *in vitro* antibody synthesis as measured by the haemagglutination method is at least as sensitive and often a more sensitive indicator of the antibody-producing capacity of tissues of immunized animals than the homotransplantation of cells from these tissues. Of course, the sensitivity of both procedures depends both upon the amounts of tissue utilized and the sensitivity of the assay for antibody.

At least 2 types of evidence are required before it can be concluded that incorporation of amino acids into antibody is related to antibody synthesis. First, it must be ascertained that incorporation is an active process which requires energy, amino acids and, perhaps, intact cells. Secondly, the radioactive material which is measured must be shown to be identical with antibody. The first of these criteria has been fulfilled by previous studies (Ogata *et al.*, 1956; Askonas and Humphrey, 1958; Wolf and Stavitsky, 1958) as well as by the present data. The second criterion has been fulfilled to some extent since the radioactive material which is measured is: (1) associated with the γ-globulin fraction of protein; (2) co-precipitated by the specific precipitate from a medium in which lymphoid tissues of immunized animals have been incubated, but not from a medium in which non-immunized lymphoid tissues or non-lymphoid tissues have been incubated; (3) not produced by the tissues of immunized animals which do not produce antibody (Expt. 22, Table V); (4) more readily demonstrable during the anamnestic response (Keston and Katchen, 1956; Askonas and Humphrey, 1958); (5) correlates roughly with antibody measured by haemagglutination; (6) produced when ^{14}C amino acids are employed so that radioactivity cannot be attributed to S-S linkage involving S-containing amino acids.

The data cited plus the failure of exchange to occur between extracellular protein and the amino acids of the medium (Askonas and Humphrey, 1958) provide strong evidence that the incorporation of amino acids into antibody is in fact a measure of genuine synthesis. It would, however, be desirable to demon-

strate the incorporation of amino acids into a peptide linkage in the antibody molecules.

There are some apparent discrepancies between the haemagglutination and isotopic assays. Some of the media (79, 88) which supported as much antibody synthesis as the W medium as measured by the haemagglutination assay sometimes supported somewhat less incorporation than on W medium. It is probably relevant that the apparent discrepancies occurred in experiments in which the tissues apparently were not synthesizing much antibody and the haemagglutination method was being employed at its lowest sensitivity whereas the isotopic method was still sensitive. In the experiments in which toxoid was employed the discrepancy between incorporation and haemagglutination may be associated to some extent with the fact that a relatively pure preparation was employed in the isotopic assay, whereas a less pure preparation was used to sensitize the erythrocytes in the haemagglutination procedure. The purity of the antigen is a less likely explanation for the other antigens, however, because the same antigenic preparations were employed in the haemagglutination and isotopic assays. A more likely explanation for the discrepancy is that some of the antibody which was measured by the isotopic method was non-precipitating. Previous studies have indicated that non-precipitating antibodies are not detectable by the haemagglutination method (Stavitsky, 1958*b*) but probably detectable by the method employed in these isotope experiments of co-precipitating any non-precipitating antibody with toxoid and precipitating antitoxin (Kuhns, 1956). There is considerable evidence suggestive of the production of non-precipitating antibody during various stages of the antibody response to various proteins (Heidelberger, Treffers and Mayer, 1940 ; Pappenheimer, 1940).

Recent evidence gives one considerable confidence in the specificity and sensitivity of the *in vitro* isotopic method for the study of antibody synthesis. In fact, it may now be stated with assurance that alterations in antibody function or the mechanisms of this process can be studied with greatest sensitivity in isolated tissues of immunized animals by the *in vitro* isotopic method. The *in vitro* method possesses other obvious advantages over *in vivo* method in that it employs less tissue, a shorter period of incubation, is subject to better control and analysis and, finally, if one biopsies part of an organ for *in vitro* study, antibody synthesis may be investigated simultaneously both *in vitro* and *in vivo*.

Among the limitations of the isotopic method as at present carried out are :

1. The small amounts of antibody produced, which may be remedied by the use of larger amounts of tissue and longer periods of incubation.

2. Rigid controls and attention to detail are needed in order to reduce the non-specific adsorption of radioactivity.

The nature of the material which is non-specifically adsorbed by the heterologous antigen-antibody precipitate is unknown. It is non-dialyzable, increases in amount with increasing time of incubation of tissue and is diminished in amount when the tissue either is damaged or incubated in the absence of amino acids (Stavitsky, unpublished). For these reasons it has provisionally been considered a protein which apparently is being synthesized by tissues under the conditions of our experiments.

With a full appreciation of its advantages and limitations it would appear that the isotopic method applied to *in vitro*-antibody-synthesizing systems is a valuable addition to the techniques applicable to the study of the basic aspects

of antibody synthesis. Preliminary attempts in this direction have been indicated in Table V. The isotopic assay has also been used successfully for the demonstration of the rapid *in vitro* induction and occurrence of both the primary and secondary antibody responses (Stavitsky, 1958*c* and *d*).

SUMMARY

Initial experiments indicated that *in vitro* antibody synthesis as measured by haemagglutination was at least as sensitive and often more sensitive a criterion of antibody-synthetic capacity of a tissue than the synthesis which occurred when cells from these tissues were transplanted to normal animals. Subsequent experiments were concerned with the relative specificity and sensitivity of the *in vitro* synthetic method compared to the isotopic assay of antibody synthesis. The isotopic assay involved the incorporation of radioactive amino acids into antibody. Evidence was obtained that incorporation was an active process requiring energy, amino acids and intact cells and, secondly, that the radioactive material produced was antibody. The data thus indicated that the incorporation method was a measure of antibody synthesis. Certain apparent discrepancies between the haemagglutination and isotopic asssays were attributed to the ability of the latter method to detect non-precipitating antibody. The material which was non-specifically adsorbed by a heterologous antigen-antibody precipitate in the isotopic assay has provisionally been considered an unknown protein which is being synthesized under the conditions of these experiments. The advantages and limitations of the isotopic method compared to other methods were discussed and it was concluded that the isotopic method is a valuable addition to the techniques used to study the basic mechanisms of antibody synthesis. Certain preliminary applications of the method were indicated.

The author is very grateful to Frank C. Williams and Jerome Golub for excellent technical assistance. He is also appreciative of the collaboration of Thomas R. Wood who carried out many of the experiments on the relationship of amino acid incorporation to antibody synthesis.

The investigation was supported by grants from the Life Insurance Medical Research Fund and from the U.S. Public Health Service.

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