Reports

Purification of Antibody to Galactosyl-Protein Conjugates

Abstract. A rapid procedure for purification of rabbit antibody to β-D-galactosylphenylazo-bovine serum albumin is described. Antibody was precipitated with a heterologous antigen and was then dissociated from the precipitated complex with hapten. The antigen was partially removed by *p*H adjustment, and antibody was separated from residual antigen and from hapten by partition chromatography on Sephadex G-25. The antibody, obtained in 50 to 55 percent over-all yield, was 96 percent reactive with homologous antigen. It appeared to consist of 95 percent of a single component, based upon physicochemical measurements.

In connection with work on biosynthesis and location of the combining regions of rabbit antibody to the β-D-galactosyl group, it became necessary to seek facile methods for the preparation of highly purified material. Several procedures which had previously been devised (*1*) for obtaining antibodies in a relatively pure state were employed, but were not applicable to this system because of poor resolution of protein and because of low over-all yields. The method reported here takes advantage of the ease of dissociation of a heterologous antigen-antibody precipitate by the hapten *p*-nitrophenyl-β-D-galactoside and uses the finding (*2*) that substances of low molecular weight may be separated from more complex molecules by chromatography on a Sephadex column. Heterologous precipitating antigens have been used previously in other procedures (*3*) for the purification of antibodies.

The immunizing antigen was prepared by coupling *p*-aminophenyl-β-D-galactoside to bovine serum albumin (BSA, Armour fraction V) by diazotization, as previously reported (*4*). Analysis of this purified conjugate by the anthrone method demonstrated that 17 sugar residues were attached per mole of bovine serum albumin. The precipitating antigen was synthesized by treating human serum albumin (*5*) in the same fashion. It contained 13 moles of bound sugar per mole of protein. The heterologous precipitating protein, under comparable conditions, removed 60 to 65 percent as much antibody from solution as the homologous antigen did.

An antiserum was obtained from rabbits immunized with alum-precipitated antigen. It contained 100 mg of antibody precipitable by galactosylphenylazo–bovine serum albumin. After absorption of this antiserum with bovine serum albumin to remove antiprotein antibodies, 14 mg of the heterologous antigen was added to the supernatant. After incubation at 37°C for 1 hour, the mixture was cooled to 4°C for 3 hours. Prolonged incubation in the cold at this point did not materially increase yields. The resultant precipitate was removed by centrifugation and was washed with phosphate buffer (*p*H, 6.8; Γ/2, 0.05) several times with thorough mechanical suspension in order to remove nonspecifically adsorbed protein. This antigen-antibody complex was then dissociated by incubating for 0.5 hour at 37°C with 2 ml of 0.05*M* *p*-nitrophenyl-β-D-galactoside. The precipitate dissolved completely. Since it was found that borate ions, when added at this point, aided in the removal of precipitating antigen, 1 ml of 0.1*M* boric acid was added, and the *p*H was lowered to 4.7 in the cold with 0.1*N* HCl. The mixture was centrifuged at 5°C at 2000 rev/min for 1 hour to separate the antigen.

A Sephadex G-25 (Pharmacia, Uppsala, Sweden) column, 30 by 2.5 cm, was prepared by addition of 25 g of the cross-linked dextran polymer to phosphate buffer (*p*H, 6.8; Γ/2, 0.05). After the column had stood overnight, it was washed with buffer until absorption in the ultraviolet at 280 mμ could no longer be detected in the washings. The supernatant solution, after removal of antigen as described above, was placed on top of the column, and the chromatogram was developed in the cold room with the phosphate buffer. Column effluent was collected in 10-ml portions, and the appearance of protein in the fractions was checked by ultraviolet absorption measurements. The antibody appeared as a sharp peak in the 70- to 90-ml volume of effluent. Residual antigen and *p*-nitrophenyl-β-D-galactoside were not eluted until several hundred milliliters of phosphate buffer had been run through the column. Complete resolution of hapten from antibody combining sites was demonstrated by examination of the fractions containing antibody for the chromogenic hapten by the usual hydrolysis and alkaline color test for *p*-nitrophenolate anion. It was not possible to detect any hapten by this procedure. After all ultraviolet-absorbing material had been removed by washing with excess buffer, the column was suitable for another chromatographic run. The total recovery of purified antibody was 50 to 55 mg in several experiments. This was an almost quantitative yield in the steps involving solubilization of antibody with hapten and removal of impurities by Sephadex chromatography.

The resultant antibody was examined by sedimentation and electrophoretic analyses (*5*). It was shown that 95 percent of the protein had a sedimentation constant of 6.45, while the remainder, probably a macroglobulin, had a constant of 18.9 *S*. The electrophoretic mobility was typical for a γ-globulin, although some spreading after 3 hours, probably due to the presence of the macroglobulin (*6*), was seen. Immunoelectrophoresis of the antibody against sheep anti-rabbit serum indicated a single component, and no non-γ-globulin impurities were detected. The addition of increasing amounts of homologous antigen to the purified antibody showed that 80 percent of the total protein was specifically precipitated at the maximum point. The supernatant from this precipitation was tested (*7*) for the presence of nonprecipitating antibody by coprecipitation with an additional known amount of antigen and precipitating antibody. Another 16 percent of the initial protein present was recovered by this procedure, so that 96 percent of the purified antibody solution could be specifically reacted with homologous antigen.

In addition to high resolution and yield of antibody, this procedure is advantageous because of the minimum number of manipulations involved.

Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should *not* repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper.

Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* **125**, 16 (1957)].

Time-consuming dialyses, which often result in a considerable degree of protein denaturation, are thus avoided. At present this method is being tested with other anti-hapten systems (*8*).

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23 January 1961

Age Changes in Adenine Nucleotides in Flight Muscle of Male House Fly

Abstract. Under standard conditions of temperature, humidity, and (artificial) diet, loss in flight ability is paralleled by a decline of up to 66⅔ percent in activity of enzymes dephosphorylating organophosphorous compounds in the thoracic flight muscle. Concomitantly, the content of adenosine triphosphate in the flight muscle increases five times, while the content of adenosine monophosphate correspondingly diminishes.

In an earlier paper, Rockstein reported (*1*) that the male house fly, *Musca domestica* L., reared and maintained under standardized conditions of temperature, humidity, and artificial diet, consistently demonstrated failure in flight function in the form of abrading and (ultimately) loss of its wings beginning with the 10th and usually by the 14th day of adult life. Concomitant biochemical changes were also observed in the form of marked decline in the activity of sodium β-glycerophosphatase activity from five activity units at 1 day to 1.8 units by the 11th day. Follow-up determinations of activity in the thoracic flight muscle of magnesium activated adenosine triphosphatase, by the method of Sacktor (*2*), have shown a remarkably similar decline in the activity of this enzyme, both in total thoracic homogenate extracts as well as in the flight muscle mitochondria per se, especially after the 9th to 10th day.

In order to explore further the biochemical manifestations of the senescence in flying ability of the male house fly, we undertook to determine what changes might be taking place in the substrate of this important enzyme system, adenosine triphosphate and its breakdown products, in the aging flight muscle of the male fly. Flies of known age of the NAIDM (*3*) strain were anesthetized lightly with CO_2, sexed, and frozen solid by rapid plunging into liquid air. For each age, 50 thoraces were isolated and ground in cold 0.6*N* perchloric acid in an Elvehjem-Potter (Teflon and glass) homogenizer. This and all other subsequent procedures preparatory to chromatographic separation were carried out at approximately 0°C. The total homogenate was transferred quantitatively to a centrifuge tube and centrifuged at 2000*g*. The supernatant liquid was set aside and stored cold, and the centrifugate was resuspended in cold 0.2*N* perchloric acid and recentrifuged. The combined supernatant liquids were then neutralized with 5*N* sodium hydroxide and transferred to a Dowex 1-X 10 (200 to 400 mesh) ion-exchange resin column (1 by 6 cm), previously prepared in the formate form, according to the Siekevitz-Potter method (*4*). By the modified Carter and Cohn gradient elution technique (*5*), we separated the three adenine nucleotides, with adenosine monophosphate and adenosine diphosphate being eluted by the distilled water–4*N* formic acid system and adenosine triphosphate by a similar formic acid–0.6*N* ammonium formate in 4*N* formic acid system (*6*). Estimation of each nucleotide was made by absorbance at 260 mμ, in the Beckman DU spectrophotometer. Standard solutions of each of these nucleotides were run through the column once weekly to check the consistency of this fractionation system. By this method, adenosine monophosphate was eluted completely in the 6th to 15th (5 ml) collecting fractions, adenosine diphosphate between the 30th to 50th tubes, and adenosine triphosphate in tubes 75 to 90.

Table 1 shows that the content of adenosine triphosphate in the thorax of the male house fly rises significantly after the 1st week and reaches a maximum value by the 9th day, that is, at a time when the enzyme system involved in its dephosphorylation approaches a minimum. Concomitantly and reciprocally, the content of adenosine monophosphate (the breakdown product of adenosine triphosphate) reaches a minimum level. These biochemical manifestations of senescence of flight ability all reflect morphological and gross functional alterations with age in flying ability at the virtually identical point in the life history of the male fly. The recognized role of phosphorus metabolism in general and the dismutation of "high-energy" bonds in the energizing of a number of important biological processes, including muscle contraction (*7*), make these correlative positive observations of a quantitative nature highly gratifying in an area where more and more concrete evidence of a fundamental biological phenomenon (like senescence) is being sought after. In order to pinpoint the actual sites of such changes within the flight muscle itself, current investigation is being made of biochemical changes and concomitant changes in the cytochemical-cytological features of the giant mitochondria (sarcosomes), the fibrillar, and the soluble fractions of the flight muscle of the aging house fly. Preliminary findings point strongly to the primary role of a declining intramitochondrial enzyme and an extramitochondrial site of the changing adenine nucleotide content (*8, 9*).

Table 1. Adenine nucleotides in the flight muscle of aging male house flies, *Musca domestica* L. Amounts are expressed in absorbance units per total extract from 50 thoraces, at each age level. AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Age	AMP	ADP	ATP
0–2 hr	3.9	1.2	1.0
6–8 hr	4.4	1.1	0.7
22–24 hr	5.7	0.8	1.6
44–48 hr	5.3	0.9	0.8
3–4 day	4.9	0.4	0.6
4–5 day	6.6	0.5	1.9
6–7 day	4.6	1.6	2.7
8–9 day	1.2	2.0	6.7
12–13 day	1.8	2.2	5.2
14–15 day	1.7	1.8	5.2
15–16 day	1.1	1.3	6.2

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