D. Y., *Quart. Bull. Northwest. Univ. Med. School,* 1944, v18, 298.

4. Verney, E. B., *Proc. Roy. Soc. London,* 1947, v135, 25.

5. Pickford, M., Watt, J. A., *J. Physiol.,* 1951, v114, 333.

6. Freedman, A. M., Bales, P. D., Willis, A., Himwich, H. E., *Am. J. Physiol.,* 1949, v156, 117.

7. Freedman, A. M., Himwich, H. E., *ibid.,* 1949, v156, 125.

8. Rinaldi, F., Himwich, H. E., *Arch. Neurol. and Psychiat.,* 1955, v73, 387.

9. Longo, V. G., *Experientia,* 1955, v11, 76.

10. Aprison, M. H., Nathan, P., Himwich, H. E., *Am. J. Physiol.,* 1956, v184, 244.

11. Bradley, P. B., *EEG Clin. Neurophysiol.,* 1953, v5, 471.

12. Sawyer, C. H., Gernandt, B. E., *Am. J. Physiol.,* 1956, v185, 209.

13. Longo, V. G., Silvestrini, B., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 43.

14. Clemente, C. D., Sutin, J., Silverstone, J. T., *Am. J. Physiol.,* 1957, v188, 193.

15. Jolly, E. R., Steinhaus, J. E., *J. Pharm. and Exp. Therap.,* 1956, v116, 273.

16. Marrazzi, A. S., Hart, E. R., *Science,* 1955, v121, 365.

17. Ginsburg, M., Brown, L. M., p109, in *The Neurohypophysis,* ed. H. Heller, Academic Press, N. Y., 1957.

18. Rudolph, A. M., Paul, M. H., *J. Appl. Physiol.,* 1957, v10, 327.

19. Diamant, H., *Acta Oto-laryngologica,* 1954, Suppl. 111, 1-84.

Received March 4, 1958. P.S.E.B.M., 1958, v98.

Immunochemical Studies with Radioactive Isotope, Similarity and Difference Between Serum Vitellin and Lipovitellin. (24157)

TSUNEO ABE, YUICHI TANABE, TADATSUNE KANEKO, KAZUSHIGE MOGI AND TATSUO HOSODA (Introduced by A. V. Nalbandov)

National Institute of Agricultural Sciences, Chiba-Shi, Japan

The serum of laying hens was first distinguished from that of non-laying hens and of cocks by Sasaki(1) who used immunological precipitin tests. Roepke and Bushnell(2) using immunological methods detected traces of protein similar to ovovitellin in serum of cocks, and more in laying hens. Lakowski (3) found in plasma of laying hens a phosphoprotein which he named serum vitellin since its chemical nature is similar to ovovitellin. Recently Hosoda and his associates(4), using immunological technic determined the vitellin content in blood serum of hens and found that its titer corresponds to ovarian activity of hens. Wormall and his co-workers (5,6) studied the chemical composition of lipovitellin-antilipovitellin precipitates using radioactive isotopes. The present work was undertaken to elucidate the relationship between serum vitellin and egg yolk lipovitellin, using immunochemical methods with radioisotope.

Materials and methods. Antigens. 350 μc P^{32} as Na_2HPO_4 was injected subcutaneously into laying hens. After 24 hours the hens were sacrificed, and P^{32}-containing serum and yolk solution taken up as antigens in 10% NaCl. *Antiserum* to serum of laying hens was prepared by immunizing rabbits with non-radioactive serum of laying hen, and absorbing the species-specific antibodies with cock serum. This type of absorbed-antiserum has been used by Hosoda *et al.*(4) for determination of serum vitellin. Anti-yolk serum was prepared by immunizing rabbits with a suspension of non-radioactive yolk. This antiserum had a considerable amount of species-specific antibodies, absorbed with cock serum. Lipovitellin used as antigen was prepared as described by Chargaff(7). Antilipovitellin serum was prepared by immunizing rabbits with a suspension of nonradioactive lipovitellin. Anti-lipovitellin serum also had considerable amount of species-specific antibodies, which were absorbed with cock serum. Phenol was added in a concentration of 0.25% to all antisera for preservation, and these antisera were stored at 3-5°C. *Precipitin tests.*

Each 1 cc of P^{32}-containing serum and 5% (W/V) solution of egg yolk in 10% NaCl, used as antigens, were mixed with 20 cc of antiserum, the mixtures were kept 2 hours at 37°C over-night at 0-4°C in refrigerator, then centrifuged 10 min. at 2-4°C at 4000 rpm. For each serological test, complete disappearance of antigen was confirmed by interfacial precipitin reactions on the supernatant solution. In further experiments, NaCl was added to each of P^{32}-containing serum and antisera to give the same salt concentration (10%) as in egg yolk solution. In another case, yolk solution in 10% NaCl was mixed with 10%-NaCl-added antisera. The succeeding procedure was the same as described above. The experiments with yolk solution were performed only to avoid the possibility of precipitation of lipovitellin by reduction in salt content; however, no precipitate was observed when 1 cc of a 5% solution of yolk in 10% NaCl was diluted with 20 cc of non-immunized serum of rabbit or cock. *Phosphorus determinations and radioactivity measurements.* All of the precipitates were washed twice with small amounts of ice cold distilled water to remove non-specific substance, and all the supernatants were fractionated by the method of Schneider(8). After the acid soluble fraction was extracted with cold trichloroacetic acid, the lipid fraction was removed by extraction with hot ethanol-ether (7:3 by vol.), and the nucleic acid fraction was extracted with hot trichloroacetic acid. The residue was regarded as the protein fraction. Lipid P and protein P in serum and yolk used as antigen were determined by the method of Fiske and Subbarow(9). Measurement of radioactivity of P^{32} in each fraction was made with a Geiger-Muller tube with a scaler. P contents in lipid fraction and protein fraction of precipitates were calculated by comparing radioactivity of fractions with that of original serum and yolk used as antigen(6,10).

Results. P^{32}-containing serum and yolk obtained from laying hens 24 hours after injection of P^{32}, were fractionated by immunological method described, into the precipitate and the supernatant. Distribution of radioactivity of P^{32} in phosphorus partitions of the precipitates, the supernatants obtained with serum of laying hen and yolk solution in 10% NaCl and various antisera to those substances containing no extra salt, is given in Table I. Antisera to the serum of laying hen absorbed with cock serum, anti-yolk serum, anti-yolk serum absorbed with cock serum, anti-lipovitellin serum, and anti-lipovitellin serum absorbed with cock serum were used. In all cases acid soluble P was not detected in the precipitates, but it was present in supernatant solutions, and in any case nucleic acid P was not detectable in precipitates and supernatants. When serum of laying hens was used as antigen, each of the absorbed antisera described above precipitated one-half of the radioactivity of P^{32} in lipid P fraction, while unabsorbed antisera precipitated two-thirds of it in the same fraction. On the other hand, approximately 95% of radioactivity of P^{32} in protein P fraction was always present in precipitates. The precipitates obtained from mixtures of sera of laying hens and from unabsorbed antisera, contained considerably more phospholipin on the basis of phosphoprotein than did precipitates obtained from mixtures of sera of laying hens, and absorbed antisera.

When yolk solution in 10% NaCl was used as antigen, each of the unabsorbed antisera precipitated three-fourths of the radioactivity of P^{32} in lipid P while precipitates obtained with absorbed antisera contained 55% of the activity. Only 22-36% of the radioactivity of P^{32} in protein P was precipitated in every case, while 95% was precipitated when serum of laying hens was used as antigen. Yolk-unabsorbed antisera precipitates contained considerably more phospholipin on the basis of phosphoprotein than did yolk-absorbed antisera precipitates.

No difference in distribution of radioactivity of P^{32} in each fraction was observed among the 3 kinds of unabsorbed antisera. The same phenomenon was observed among absorbed antisera.

Composition of precipitates obtained from mixtures of serum and yolk of laying hens, and of various antisera to these substances, is

TABLE I. Distribution of Radioactivity of P^{32} in Phosphorus Partitions of Precipitates and Supernatants Obtained with Serum and Yolk* of Laying Hen and the Various Antisera† to These Substances.

Antigen fractions	Antiserum to laying hen serum		Absorbed‡ antiserum to laying hen serum		Anti-yolk serum		Absorbed‡ anti-yolk serum		Anti-lipovitellin serum		Absorbed‡ anti-lipovitellin serum	
	ppt.§	sup.§	ppt.	sup.	ppt.	sup.	ppt.	sup.	ppt.	sup.	ppt.	sup.
Serum												
Acid soluble ph.‖	1.9 (19)	7.5 (81)	.2 (1.2)	14.6 (98.8)	1.1 (6.7)	15.3 (93.3)	.7 (4.2)	15.9 (95.8)	.8 (5.5)	13.8 (94.5)	.4 (2.6)	15 (97.4)
Lipid ph.	36.2 (60.5)	23.6 (39.5)	29.3 (50.8)	28.4 (49.2)	33.1 (63.8)	18.8 (36.2)	24.8 (45.8)	29.3 (54.2)	39.1 (71.3)	15.7 (28.7)	29.4 (55.2)	23.9 (44.8)
Nucleic acid ph.	0	0	.6	0	.8	.2	1.1	.1	.9	0	.9	.3
Protein ph.	30.0 (97.7)	.8 (2.3)	25.3 (94)	1.6 (6)	29.6 (96.4)	1.1 (3.6)	26.7 (95)	1.4 (5)	28.8 (97.3)	.8 (2.7)	28.9 (96)	1.2 (4)
Total	68.1	31.9	55.4	44.6	64.6	35.4	53.3	46.7	69.7	30.3	59.6	40.4
Yolk												
Acid soluble ph.	0	5.1	0	7.7	0	8.5	1.7	9.3	.2	7.9	.7	9.6
Lipid	48.2 (71.1)	19.6 (28.9)	38.7 (56.8)	29.4 (43.2)	50.2 (75.1)	16.6 (24.9)	33.4 (51.4)	31.6 (48.6)	52.3 (80.6)	12.6 (19.4)	40.1 (63.8)	22.7 (36.2)
Nucleic acid ph.	0	0	0	0	.3	0	0	.6	1	.8	.4	0
Protein	7 (26)	20 (74)	8.7 (36)	15.5 (64)	5.4 (22.1)	19 (77.9)	6.6 (28.1)	16.9 (71.9)	6.2 (24.7)	18.9 (75.3)	8.2 (30.9)	18.3 (69.1)
Total	55.2	44.7	47.4	52.6	55.9	44.1	41.7	58.4	59.8	40.2	49.4	50.6

* Solution in 10% NaCl. † Not containing any extra salt. ‡ Absorbed with cock serum.
§ ppt. = precipitate; sup. = supernatant. ‖ ph = phosphorus.
Figures in parentheses refer to percentage composition in each fraction.

shown in Table II. The ratio lipid P : protein P in the precipitates obtained with sera of laying hens and the absorbed antisera was 1.1:1, and 1.4:1 in those obtained with sera of laying hens and unabsorbed antisera. This ratio was 4.5:1 in the yolk-absorbed antisera precipitates, and 8:1 in yolk-unabsorbed antisera precipitates.

When NaCl was added to sera of laying hens and to antisera to a concentration of 10%, lipid P content of precipitates diminished to 60% of precipitates obtained with sera and antisera which did not contain extra salt. Protein P content of precipitates obtained from mixtures of yolk solution in 10% NaCl and the 10% NaCl-added antisera, showed a similar tendency. A reduction of lipid P content to 70-80% of that of precipi-

TABLE II. Composition of Precipitates Obtained with Serum and Yolk of Laying Hens and Various Antisera to These Substances.

Antigen			Antisera											
			Antiserum to laying hen serum		Absorbed* antiserum to laying hen serum		Anti-yolk serum		Absorbed* anti-yolk serum		Anti-lipovitellin serum		Absorbed* anti-lipovitellin serum	
	LP	PP	LP	PP	LP	PP	LP	PP	LP	PP	LP	PP	LP	PP
Serum	68 (1.6)	88 (1)	96.5 (1.2)	81.7 (1)	84.0 (1.1)	75.1 (1)	106.5 (1.3)	80.0 (1)	82.0 (1.1)	74.0 (1)	127.5 (1.6)	79.0 (1)	92.8 (1.2)	76.5 (1)
Yolk	172 (2.5)	68 (1)	122.3 (6.9)	17.7 (1)	102.0 (4.4)	23.3 (1)	117.2 (8.4)	14.0 (1)	80.5 (4.8)	16.9 (1)	126.0 (8.0)	15.8 (1)	86.3 (4.6)	18.8 (1)

* Absorbed with cock serum. LP = Lipid phosphorus; PP = Protein phosphorus.
Figures in parentheses showed ratio lipid P : protein P in precipitates. All weights are terms of μg per cc of antigen used (serum: 1 cc, yolk: 1 cc of 5% (W/V) egg yolk solution).

tates obtained with yolk solution in 10% NaCl and antisera containing no extra salt was observed.

Discussion. The data showed that each antiserum to serum of laying hens (absorbed with cock serum and unabsorbed), to yolk (absorbed and unabsorbed), and to lipovitellin (absorbed and unabsorbed) precipitated 95% or more of protein P in serum of laying hens, but only about 25% of that in the yolk dissolved in 10% NaCl. It is noted that phosvitin is dissociated from yolk lipovitellin in 10% NaCl solution, and is recombined with lipovitellin when it is reprecipitated(11). Mecham and Olcott(12) and Francis(11) stated that 75% of protein P of vitellin appeared to be due to phosvitin. Furthermore this substance has been shown not to react with antisera to lipovitellin or to vitellin(11). It may be due to the presence of phosvitin dissociated from lipovitellin in 10% NaCl solution, that only 25% of protein P in yolk solution in 10% NaCl can be precipitated with the antisera. However, it seems less probable that in serum of laying hen phosvitin combines with serum vitellin because the present experiments demonstrated that, when salt was added in 10% concentration to serum of laying hens, the antisera still precipitated 95% of protein P in the serum. In other words, no dissociation of phosphoprotein from serum vitellin was observed. Possibly there exists some difference between the structure of serum vitellin and that of lipovitellin in egg yolk, and the serum vitellin may be chemically different after passage through the granulosa of the follicle.

In this study, it was observed that unabsorbed antisera precipitated considerably more phospholipin on the basis of phosphoprotein than did antisera absorbed with cock serum, regardless of whether the serum of laying hens or egg yolk was used as antigen. These results led to the interpretation that unabsorbed antisera precipitate phospholipo-P-free or nearly free protein present in serum of laying hens, which has a similar antigenicity to the substance present in cock serum.

The present study showed that the mean ratio of lipid P to protein P was 1.1:1 and 1.4:1, in precipitates obtained with sera of laying hens and absorbed antisera and those obtained with sera of laying hens and the unabsorbed antisera, respectively. This ratio becomes 4.5:1 in yolk-absorbed antisera precipitates, and 8:1 in yolk-antiyolk or antilipovitellin precipitates. Banks *et al.*(6) reported that the ratio lipid P to protein P in the lipovitellin-antilipovitellin precipitates was 5:1, compared with 1.6:1 for the sample of lipovitellin used. The difference between the precipitates and lipovitellin used as antigen observed by them may be also ascribed to the dissociation of phosvitin having no antigenicity.

The present study demonstrated that injection of serum of laying hens, egg yolk, and lipovitellin into the rabbit produced an antibody which precipitated the same kind of lipoprotein as far as phosphorus is concerned. After the species-specific antibody was excluded, the remainder of the antibody in each case, precipitated whole phosphoprotein combining phospholipin in serum of laying hens; however, in a solution of yolk in 10% NaCl, it appeared to precipitate only non-phosvitin phosphoprotein combining phospholipin. Thus, it is concluded that the structure of phosphoprotein (serum vitellin) combining phospholipin, present in serum of the laying hen, is similar to that of lipovitellin in egg yolk, though there is some difference between them.

Francis and Wormall(5) reported that both lipovitellin and lipid-free vitellin injections produced antisera capable of precipitating lipovitellin. Furthermore the specific antigenic substance in this reaction is greatly affected at 70°C, and completely destroyed at 80°C(13). These results strongly suggest that the antigenicity of lipovitellin may be due to the protein component of the substance. However, phosvitin contained in lipovitellin and accounting for 75% of protein P of vitellin, has been shown not to react in precipitin reactions with anti-lipovitellin serum or antivitellin serum. Injection of phosvitin into rabbits does not produce sera capable of reacting in precipitin reactions with phosvitin, or lipovitellin(11). The antigenicity of lipo-

vitellin would therefore appear to be due to the non-phosvitin component of the protein.

Summary. With the help of radioisotope, immunochemical studies on the similarity and differences between serum vitellin and yolk lipovitellin, were made. When P^{32}-containing serum of laying hens was used as antigen, each antiserum to the serum of laying hens (absorbed with cock serum and unabsorbed, to yolk, absorbed and unabsorbed, and to lipovitellin, absorbed and unabsorbed), precipitated 95% of the radioactivity of P^{32} in protein P fraction, and 50-65% in lipid P fraction. On the other hand, when P^{32}-containing yolk solution in 10% NaCl was used as antigen, these antisera precipitated only 22-36% of radioactivity in the former fraction, and 55-80% in the latter fraction. The ratio of lipid P to protein P in the precipitates obtained with the sera of laying hens and the above described antisera was 1.1-1.6:1, and, in those obtained with yolk and the antisera was 4.5-8:1, compared with 1.6:1 for the lipovitellin used. Injection of the serum of laying hens, yolk and lipovitellin into rabbits produced antibodies which precipitated a similar lipoprotein.

We wish to thank Dr. Kazuo Kuretani, Scientific Research Institute, Tokyo, for his great help and valuable advice.

1. Sasaki, K., *J. Immunol.*, 1932, v23, 1.
2. Roepke, R. R., Bushnell, L. D., *ibid.*, 1936, v30, 109.
3. Laskowski, M., *Biochem. J.*, 1935, v278, 345.
4. Hosoda, T., Kaneko, T., Mogi, K., Abe, T., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 502.
5. Francis, G. E., Wormall, A., *Biochem. J.*, 1948, v42, 469.
6. Banks, T. K., Francis, G. E., Mulligan, W., Wormall, A., *ibid.*, 1951, v48, 180.
7. Chargaff, E., *J. Biol. Chem.*, 1942, v142, 491.
8. Schneider, W. C., *ibid.*, 1945, v161, 293.
9. Fiske, C. H., Subbarow, Y., *ibid.*, 1925, v66, 375.
10. Chargaff, E., *ibid.*, 1942, v142, 505.
11. Francis, G. E., *Biochem. J.*, 1952, v51, 715.
12. Mecham, D. K., Olcott, H. S., *J. Am. Chem. Soc.*, 1949, v71, 3670.
13. Hosoda, T., Kaneko, T., Mogi, K., *Jap. J. Zootech. Sci.*, 1952, v22, 75.

Received April 8, 1958. P.S.E.B.M., 1958, v98.

Preferential Reduction of Conjugated Bilirubin to Urobilinogen by Normal Fecal Flora.* (24158)

C. J. WATSON, MALCOLM CAMPBELL AND PAUL T. LOWRY
Department of Medicine, Univ. of Minnesota Medical School and Hospital, Minneapolis

In earlier experiments in which crystalline bilirubin was added to broth cultures of normal fecal bacteria, it was evident that the proportion reduced to urobilinogen was quite small(1). The possibility existed that these poor conversions depended upon use of free, non-polar bilirubin rather than polar bilirubin glucuronide, the conjugated form of bilirubin present in the bile(2). To examine this possibility the following experiments have been carried out.

Methods. A crude concentrate of bilirubin glucuronide was prepared as follows: Fresh human fistula bile was collected in a container having sufficient saturated ammonium sulfate solution to provide at least 50% saturation at end of collection. The vessel was agitated from time to time during collection to allow mixture and precipitation of the pigment. Bile and ammonium sulfate mixture was then filtered through a large amount of Hyflo (diatomaceous silica)† on a Buchner funnel, the Hyflo first having been packed on coarse filter paper with water. Hyflo was also mixed with bile and ammonium sulfate solution before it was poured onto the funnel and the packed Hyflo on funnel was moistened with saturated ammonium sulfate solution before mixture was applied. The packed material

* Aided by contract with Surgeon General's Office, U. S. Army.

† Johns-Manville Hyflo-Supercel.