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.

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

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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.

was repeatedly washed with 50% ammonium sulfate solution. With some samples of bile, a gummy mass was noted at this stage, which was much more difficult to handle. The material on the Buchner funnel was packed fairly dry and extracted repeatedly with small volumes of acetone to remove most of the water. The entire mass was then transferred to a large mortar where it was ground successively, first with small amounts of acetone, next ethyl ether and finally chloroform. The latter removes considerable bilirubin, presumably a fraction which has become deconjugated during above procedure. The final dry powder consists mainly of Hyflo with adsorbed bilirubin glucuronide. Traces of bile acids and other impurities, including biliverdin, are also present. Most of the bilirubin adsorbed on the Hyflo is of the prompt direct type, reacting with the diazo reagent within one minute. The bilirubin glucuronide is readily removed from Hyflo simply by elution with water. After hydrolysis of its azopyrryl compound in 10% HCl for one hour at 100°, and paper chromatography, glucuronic acid was readily demonstrated by the method of Dische (3). The Hyflo powder with adsorbed bilirubin glucuronide keeps well at 4°, bilirubin concentration remaining essentially unchanged over long periods.

In a number of experiments the above described bilirubin glucuronide was compared with crystalline bilirubin in respect to ability of normal fecal flora to reduce it to urobilinogen in broth cultures. Varying amounts of broth, fecal inoculum and bilirubin were used (Table I). The bilirubin was either crystalline, or crude glucuronide as above described or as present in fresh bile. The media consisted of 10 g yeast extract/liter. The fecal inoculum was first incubated with broth for varying periods, usually 12 hours, in sidearm Erlenmeyer flask evacuated by ordinary water pump. As seen in Table I, Exp. 5 A, B and C, there is some evidence that preliminary incubation for 6-12 hours before adding bilirubin, promoted its reduction. pH was adjusted to 7.5 to 8.0 by addition of 10% NaOH, after which the bilirubin solution was added, the flask reevacuated and incubated for 24 hours. pH was readjusted with NaOH about midway in this period, then adjusted to 4-5 (acetic) after which the entire broth culture was extracted 3 times with petroleum ether (B.P. 30-60). The filtered, pooled petroleum ether was subjected to I_2H_2O method previously described(4) for conversion of urobilinogen to urobilin and extraction.‡ Total urobilin group was transferred, as the hydrochloride, to $CHCl_3$. The naturally occurring urobilin group includes varying proportions of levorotatory (l-) Stercobilin, inactive (i-) Urobilin, and dextrorotatory (d-) Dehydro-Urobilin. The problem of terminology will be considered in more detail in a separate communication. The abbreviations l-S, i-U and d-DU are employed to designate the 3 non-isomeric compounds above. Total urobilin was determined in Evelyn colorimeter with a 490 filter against standard plot of optical density of (l-) stercobilin and (i-) urobilin, which have essentially similar absorption. A sufficient volume to provide 200 mg. was then concentrated to dryness on water bath, the residue dissolved in methyl alcohol and this solution subjected to ferric chloride oxidation as described elsewhere. It suffices to note here that the reaction mixture is adjusted to pH 4.0 with sodium acetate solution, and extracted repeatedly with ethyl ether. The latter is washed repeatedly with water, the water washes being returned to the aqueous phase. The combined aqueous solution contains much of whatever l-S was initially present, as this is relatively stable while products of oxidation of the i- and d- compounds remain in the ether. They are readily extracted with 1.5 N HCl the spectral distribution curve of which is then recorded with Beckman DK spectrophotometer.

In the $FeCl_3$ reaction i-U yields a mixture of mesobiliviolin-mesobilirhodin (abs. max. 560 mμ) with smaller amounts of glaukobilin (abs. max. 650 mμ); d-DU yields mainly glaukobilin; l-S neither, its original absorption at 492 mμ easily recognizable. The approximate composition of initial urobilin

‡ With certain samples of feces, only indol and no urobilinogen was observed.

TABLE I. Urobilinogen Formation from Three Bilirubin as Contrasted with Bilirubin Glucuronide, in Broth Cultures of Fecal Flora.

Exp. No.	Feces inoculum used: g	No.	Approx. % l-S	Approx. % i-U	ml broth	Amt (mg) & type bilirubin or urobilin added	Hours of incubation: Before adding bilirubin	Hours of incubation: After bilirubin	Total urobilin group, mg	Approx. %: d-DU	Approx. %: i-U	Approx. %: l-S
1 A	1.	I	70	30	800	0	12	24	.09			
B	"		"	"	"	62 B. G.	"	"	21.06	80	20	
C	"		"	"	"	" cryst. B.	"	"	14.10	75	25	
2 A	.5	II	"	"	200	0	"	"	.04			
B	"		"	"	"	20 B. G.	"	"	8.83	100		
C	"		"	"	"	" cryst. B.	"	"	.5	20	40	40
D	"		"	"	"	" (fresh bile)	"	"	10.6	40	60	
3 A	"	III	85	15	"	10 B. G.	"	"	3.5		10	90
B	"		"	"	"	" cryst. B.	"	"	4.1			100
4 A	.25	IV	70	30	"	" B. G.	8	"	.46	100		
B	"		"	"	"	" cryst. B.	"	"	2.7	"		
C	"		"	"	"	" B. G.	"	"	.17	60	40	
5 A	.5	V	65	35	"	20 "	0	"	1.9	75	25	
								48	1.84	"	"	
B	"		"	"	"	10 "	6	24	3.1	not determined		
C	"		"	"	"	*Idem*	12	"	4.7		80	20
D	"		"	"	"	" + 200 bile salts	"	"	2.3		100	
E	"		"	"	"	10 cryst. B. + 200 bile salts	"	"	.2	60	30	10
6 A	1.	V	"	"	800	50 B. G.	"	"	16.5	100		
B	"		"	"	"	" cryst. B.	"	"	5.7	"		
C	"		"	"	"	" (fresh bile)	"	"	19.0	"		
7 A	.5	VI			200	10 B. G.	17	"	3.0		40	60
B	"				"	" cryst. B.	"	"	1.7		40	60
8 A	"	VII			"	" B. G.	19	"	3.3			100
B	"				"	" cryst. B.	"	"	1.9			100
9 A	"	VII			"	" " i-U	17	"	1.6		10	90
B	"				"	" " d-DU	"	"	3.4		15	85
10 A	.25	VIII	100	0	100	5 B. G.	14	"	1.44		50	50
B	.5				"	" "	"	"	2.38		40	60
C	.25				"	" cryst. B.	"	"	1.22		20	80
D	.5				"	" "	"	"	.98		40	60
E	"				"	0	"	"	.097		15	85
11 A	.25	IX	100	0	"	5 B. G.	15	"	1.73		30	70
B	.5				"	" "	"	"	1.96		40	60
C	.25				"	" cryst. B.	"	"	1.08		30	70
D	.5				"	" "	"	"	1.74		"	"
E	"				"	0	"	"	.088			100
12 A	.25	X	30	70	"	5 B. G.	16	"	1.37		100	
B	.5				"	" "	"	"	1.17		"	
C	.25				"	" cryst. B.	"	"	.94		90	10
D	.5				"	" "	"	"	1.15		100	
E	"				"	0	"	"	.148		90	10
13 A	.25	XI	80	20	"	5 B.G.	"	"	2.04	100		
B	.5				"	" "	"	"	1.76	"		
C	.25				"	" cryst. B.	"	"	.57	"		
D	.5				"	" "	"	"	.46	"		
E	"				"	0	"	"	.02		25	75

B. G. = bilirubin glucuronide (see text); cryst. B. = crystalline bilirubin (Armour or Eastman); i-U = inactive urobilin; d-DU = d-Dehydrourobilin; l-S = Stercobilin.

group subjected to $FeCl_3$ oxidation is determined by the 2 ratios:

$$\frac{\text{abs. } 492\ (1-S)}{\text{abs. } 560 + 650\ (i-U + d-DU)};$$

$$\frac{\text{abs. } 560\ (i-U > d-DU)}{\text{abs. } 650\ (d-DU > i-U)}$$

This method generally provides reasonably accurate information as to initial proportions and values have usually been in agreement with optical activity measurements.

Results. Bacterial formation of urobilinogen group from bilirubin glucuronide was usually more efficient than from crystalline bilirubin (Table I). In 4 experiments (3. 10A, C. 11B, D. 12B, D) the results were regarded as equivocal, although in 3 of these the amounts were slightly larger with conjugated than with free bilirubin. In but one instance (Exp. 4) was the yield clearly greater with free bilirubin. The basis for this difference is not clear. Analysis§ of the comparable experiments in Table I reveals a highly significant difference in favor of the glucuronide ($P<0.03$).

Under above conditions, bacteria from normal feces containing l-S and i-U in varying proportion, at times reduce bilirubin only or mainly to d-DU, again a mixture of d-DU, i-U and l-S is observed. A spectrogram of such a result is seen in Fig. 1. In still other cultures, the i- and l-compounds are formed (Table I). The frequent formation of d-DU by a normal fecal flora supports the view(5,6, 7) that it is a normal product of bacterial reduction of bilirubin in the colon. This is also in accord with the earlier observation(8) that d-DU is readily reduced to i-U, and the present observation (Exp. 9) that normal fecal flora, under above conditions, readily reduce d-DU to i-U and l-S. Starting with bilirubin, bacterial reducing activity may be sufficient to produce only d-DU with small amounts of i-U and l-S (Fig. 1). Starting with d- or i-, however, the reduction is often carried further to the l-form. This is well shown in Exp. 8 and 9 in which it is evident that the fecal flora reduced the added bilirubin to l-S, the added

§ Courtesy of Dr. J. E. Bearman, Div. of Biostatistics, School of Public Health, Univ. of Minnesota.

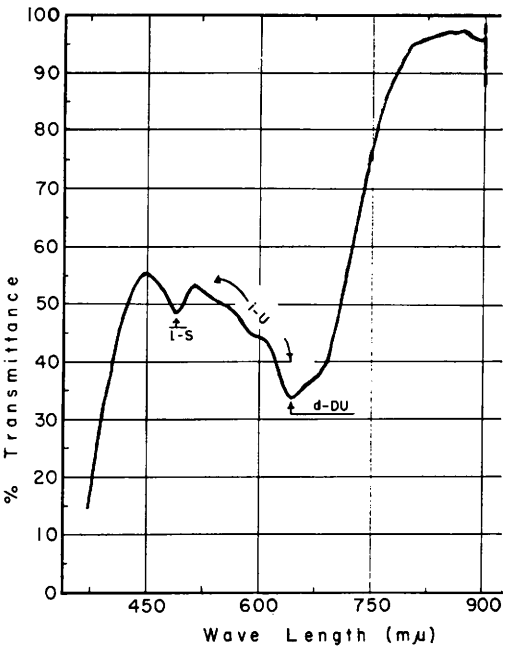

FIG. 1. Spectral distribution of products of $FeCl_3$ oxidation of urobilin group obtained by bacterial reduction of bilirubin glucuronide. Areas of absorption indicated by the arrows relate for l-S to unchanged Stercobilin, for i-U to Mesobiliviolin-Mesobilirhodin, for d-DU to Glaucobilin.

d- or i- forms mainly to l-S. In the one experiment (No. 5) with added bile salts, it appeared that there was hindrance rather than enhancement of conversion to urobilin.

Discussion. The basis for greater efficiency of bacterial reduction of the glucuronide as contrasted with free bilirubin has not been determined. It may reside simply in the polar character of the former compound, but the possibility must also be considered that the reduction is incidental to a primary attack on the glucuronic acid.

The present results emphasize bacterial formation of naturally occurring forms of urobilin(ogen) group and close interrelation of d-DU (H_{40}); i-U (H_{42}) and l-S (H_{46}). These designations avoid the objection that use of prefixes d-, i- and l-, as applied elsewhere(5) to the single term urobilin, is unconventional for compounds that are not isomers. The designation d-DU recognizes that this compound is a dehydrourobilin, as shown by our analytical data(6), and emphasized by Gray(7) and Siedel(8). Furthermore, Gray(9) has

recently described a dextrorotatory isomer of i-U, and a racemic DU. While no evidence has yet been presented for their occurrence in nature, their existence enforces the need of distinctive terminology.

Certain additional results given in Table I require brief discussion. In Exp. 2, yield from fresh bile was even greater than from crude glucuronide, yet in Exp. 5 addition of bile salts, either to crystalline bilirubin or crude glucuronide was inhibitory rather than enhancing in its effect. In general, better yields were obtained when there was a 12 hour preliminary growth of bacteria in broth, prior to addition of bilirubin. This is seen most clearly in Exp. 5, Table I.

Recent experiments carried out with S. Gilbertsen, to be described separately, indicate that at least in the normal individual there is a similar basis for failure to note significant increase of fecal urobilinogen after intraduodenal administration of free bilirubin. However, in patients with acholic feces, feeding of above described bilirubin glucuronide has thus far failed to result in formation of appreciable amounts of fecal urobilinogen and other factors must be sought to explain this discrepancy. These studies are being extended.

Summary and conclusions. 1. A method of preparing a relatively stable concentrated adsorbate of conjugated bilirubin (glucuronide) from bile, is described. This has been used in a series of experiments in broth cultures of fecal bacteria. 2. A significant and often much greater increase of urobilinogen formation was observed after addition of the conjugated as contrasted with free bilirubin. 3. The urobilinogen group formed in the bacterial cultures consisted of varying proportions of d-Dehydrourobilin, i-Urobilin, and l-Stercobilin. Under the same conditions the fecal flora readily reduced d-DU to i-U, and i-U to l-S.

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Effect of Route of Injection on Distribution and Excretion of Cortisol-4-C^{14} in the Rat. (24159)

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Distribution and metabolism of cortisol-4-C^{14} in rats has been traced after administration by intravenous, intramuscular, subcutaneous, intragastric, and sublingual routes(1,2, 3). Only minor differences in total amount excreted have been found over periods of 1 to 5 days. A previous report(4) described the fate of cortisol-4-C^{14} in the rat during the first hour after intracardiac injection. In normal animals essentially all of the injected radioactivity was recovered in the GI tract and excreta after 60 min. Extension of these studies in which the hormone was injected intravenously, showed a striking difference from previous results, a difference in distribution as well as a slower rate of excretion. This observation was elaborated since it seemed to have application in the study of the role of