sistent with an hypothesis that a polyanion essential for maintenance of ATP in aerobic homogenates of liver is made unavailable by high molecular weight polycations. The combination of large molecular weight and frequent positive charges seems essential for activity since (a) partial hydrolysis of protamine by trypsin lowers inhibitory activity, (b) bovine serum albumin exceeding protamine in molecular weight but containing few positive charges is not inhibitory, and (c) compounds such as putrescine, spermidine, spermine, and arginine comparable to protamine as bases but of relatively low molecular weight are not inhibitory. Apparently TEM under the conditions studied does not alkylate enough groups to reduce the effectiveness of the essential polyanion.

Summary. Conversion of mevalonic acid to non-saponifiable material by rat liver homogenates under conditions where conversion is a function of ATP level existing at time of mevalonic acid addition was inhibited markedly by preincubation with protamine or polylysine. Inhibition was less or absent following preincubation with trypsinized protamine, bovine serum albumin or a number of small molecular weight bases. The results are interpreted as indicating that a *combination* of high molecular weight and strong basicity are essential for inactivation of a polyanion previously shown to be essential for maintenance of ATP levels in liver homogenates.

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Investigation of Rose Bengal Conjugation.* (25939)

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Rose Bengal (tetraiodotetrachlorfluorescein) is one of the many dyes utilized in evaluation of liver function. Although uptake, storage and excretion of Rose Bengal I^{131} by the liver have been studied extensively, little is known of the metabolic processes involved. Clearance of Rose Bengal by the liver is impaired by concurrent administration of bromsulfalein (BSP)(1-4), and possibly by bilirubin(5). Recent studies have shown that hepatic clearance of BSP and of bilirubin involves conjugation with cysteine/glutathione (6,7) and glucuronic acid(8-10), respectively. Such observations suggest that these pigments may compete with Rose Bengal for initial uptake or subsequent metabolism by the liver and therefore that the competition may take place at a conjugation step. Attempts to demonstrate the presence of conjugates of Rose Bengal in biological fluids constitute the subject of this report.

Methods. Fresh human bile was obtained from 3 subjects with presumably normal liver function by means of T-tubes inserted in the common bile duct. After collection of control samples, 6-10 ml of Rose Bengal (Matheson Co.) (8.9 mg/ml) was administered intravenously; bile was collected at 30 min. intervals for 2 hr thereafter. For isotopic studies, 0.1 mC of Rose Bengal I^{131} (Robengatope, Squibb) was added to each dose before injection. Serum and urine samples were obtained from an additional subject with obstructive jaundice 45 min after injection of Rose Bengal. Control specimens were prepared by adding Rose Bengal *in vitro* to bile, serum and urine in concentrations approximating those

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TABLE I. Mobility of Pure and Secreted Rose Bengal after Paper Chromatography.

Solvent	Ratio v/v	R_f		
		Rose Bengal bile	Control bile*	Standard*
Tert butanol/water	5.9 :1	3	3	3
	4.7 :1	14	13	14
	3.7 :1	18	18	18
	2.6 :1	32	32	32
	1.72:1	64	63	64
	1.68:1	67	67	67
	1.1 :1	81	81	80
	.78:1	100		100
Tert butanol/water (ammonia atmosphere)	1.9 :1	90	90	90
Tert butanol/water (acetic acid atmosphere)	1.9 :1	50	50	50
n-butanol/ethanol/water/NH_4OH	5:.05:1:2.2	65	64	69, 42
n-butanol/acetic acid/water	4:1:2.7	100		100
1% NH_4OH saturated with isoamyl alcohol		61	59, 38	63, 38
25% ethanol/5% NH_4OH	1:1	70	72	82
Methanol/water	1.63:1	70	74, 70	83, 74

* Second value = secondary band.

found in the experimental samples. All samples were frozen and stored at −10°C until used. Rose Bengal was extracted and extraneous proteins were precipitated from bile, urine and serum by the addition of 2 volumes of acetone. As an alternate procedure on bile samples, 0.1 N silver nitrate was used as a precipitant and the pigment was redissolved in a concentrated sodium chloride solution. Bile was initially purified in paired alumina columns (1 x 5 cm). Activated alumina (Alcoa) was suspended in 90% isopropyl alcohol containing 0.13% ammonium acetate and packed by gravity. Acetone extracts of bile dried *in vacuo* were dissolved in a minimum volume of the packing solution and added to the columns. Rose Bengal was eluted in 80% isopropyl alcohol/NH_4OH (200:1). Bile extracts containing Rose Bengal I^{131} were initially purified in similar paired alumina columns using acetone/water (20:1 and 10:1) for loading and acetone/water (8:1 and 7:1) for elution of pigment. Ascending paper chromatography was performed on all samples before and after deproteination and on bile at each step of purification, using Whatman 3 MM paper and various solvent systems as listed in Table I. Mobilities of Rose Bengal from experimental and control fractions were compared by visible color and by fluorescence with a short wave ultraviolet Woods lamp (model SL 2537, Ultraviolet Products, Inc., San Gabriel, Calif.). Chromatograms of Rose Bengal I^{131} were cut into 1 cm strips which were counted in a well-type scintillation counter. Paper electrophoresis, using 0.25 M acetic acid or 0.25 M ammonium hydroxide, was performed on acetone-eluted column fractions from bile. To study the nature of Rose Bengal concentrated in the liver, 0.6-1.6 mg of the dye per 100 g of body weight was injected into the inferior vena cava of anesthetized rats. Animals were sacrificed 10-20 min after injection, the liver removed, and the pigment extracted by homogenizing the tissue in 5 volumes of acidified methylethyl ketone. Lipids were removed on the column by ether and toluene, and pigment was eluted in the isopropyl-alcohol system. Similar extracts, prepared by homogenizing the tissue in acetone/water were subjected to paper chromatography. Mercaptide conjugation was investigated in the perfused isolated liver system described by Brauer, *et al.*(11) and by Plaa(12). After a 1 hr equilibration period, 51 μC of cystine-S^{35} was added to the perfusion media; bile samples were taken at 30 min intervals. One hr later, 23.2 mg of Rose Bengal was added; bile collection was continued. Control and experimental samples, each containing an equal number of counts per total volume, were extracted and purified in paired alumina columns. Column fractions of Rose Bengal bile, purified by successive

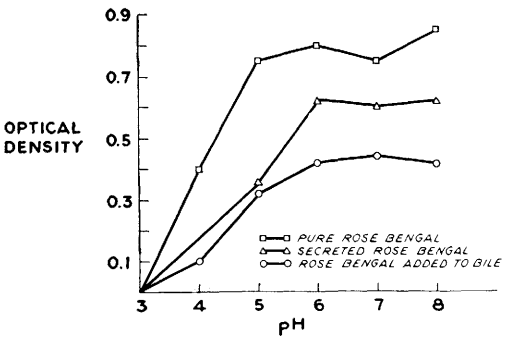

FIG. 1. Effect of pH on absorption of pure and secreted Rose Bengal at 545 mμ (unequal dye concentrations were used).

paper chromatography, were tested against internal standards for glucuronides by the Dische reaction(13).

Results. Absorption spectra at 545 mμ of pure Rose Bengal were obtained at pH 3-8 in citric acid-disodium phosphate buffers (Fig. 1). Absorption increased with increasing pH until about pH 5. The pH of maximum absorption of secreted Rose Bengal in purified bile fractions was displaced to pH 6. Identical displacement, however, was observed with control samples of bile containing added Rose Bengal.

Chromatography and electrophoresis. Secreted Rose Bengal in bile moved primarily as a single, visible fluorescent spot which could not be differentiated by extensive combinations of chromatographic systems (Table I) or by electrophoresis from Rose Bengal added to bile *in vitro*. When bile obtained after administration of Rose Bengal I^{131} was chromatographed in 6 different *tert* butanol solvents, mobilities of the visible pigment and radioactivity correlated well over a wide range of R_f values.

Rose Bengal in serum, urine and liver. Rose Bengal obtained from the serum and urine of a subject with biliary obstruction or extracted from rat liver was indistinguishable from pigment added *in vitro* to comparable control specimens.

Conjugation with cysteine or glucuronic acid. Rose Bengal and isotope appeared rapidly in bile following perfusion with cystine-S^{35} and dye. After purification by column and paper chromatography, the Rose Bengal fraction from both experimental and control bile contained only trace amounts of label. Determination of glucuronide content of highly purified extracts of bile showed that not more than 14% of the Rose Bengal could have been conjugated with glucuronic acid.

Discussion. The fact that secreted Rose Bengal was indistinguishable from pigment added to control bile and that significant levels of non-pigmented products were not observed after chromatography of secreted Rose Bengal I^{131} indicates that the pigment need not be metabolized on its passage through the liver. It is unlikely that metabolites of Rose Bengal are formed but subsequently degraded in bile since none could be detected either in the serum of a patient with an obstructed common bile duct, where regurgitation of the metabolites would be expected to occur, or in extracts from rat liver obtained shortly after injection of the dye. Under both circumstances, BSP metabolites have been reported (14). It is emphasized, however, that the negative nature of these results does not rule out the possibility that secreted Rose Bengal is modified but that the modified form could not be detected by the chromatographic systems employed in this study.

In contrast to BSP(6), cystine-S^{35} was not incorporated into secreted Rose Bengal. Therefore, competition of the 2 dyes for excretion does not occur at the step of mercaptide conjugation in the liver. Also, failure to discover a Rose Bengal glucuronide suggests that the pigment does not compete with bilirubin at the level of glucuronide conjugation.

Uptake and excretion of pigments by the liver may involve 2 independent mechanisms (15,16). Recent experiments as yet unpublished have shown that BSP is taken up and concentrated in the liver as the free pigment prior to its ultimate conjugation as a mercaptide. Conceivably, competition of various pigments may occur at this early uptake step (17) and thus be comparatively independent of the routes required for subsequent conjugation.

Summary. 1. Metabolites of Rose Bengal could not be demonstrated in human bile, serum or urine or in extracts from rat liver by a variety of systems involving paper and column chromatography and electrophoresis.

2. Conjugation with cystine-S^{35} or glucuronic acid was not demonstrable. 3. It was concluded that Rose Bengal is not conjugated by the liver in the same form reported for bilirubin (glucuronides) or bromsulfalein (mercaptides).

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Synthesis and Some Pharmacological Properties of a Peptide Derivative of Oxytocin: Glycyloxytocin.* (25940)

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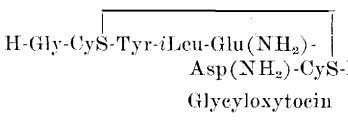
In a series of studies on the relationship between structure and biological activities of the posterior pituitary hormones and their analogs and derivatives, we became interested in the biological properties of peptide derivatives of these hormones, *i.e.* compounds in which the hormones remain unchanged except for the attachment of additional amino acids at the N- or C-terminal position. As a comparatively simple derivative of this type in which the amino group of oxytocin is involved in peptide linkage, glycyloxytocin has been synthesized.



Glycyloxytocin

Method of synthesis. The protected decapeptide, carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide, which was employed as the intermediate for the glycyloxytocin, was prepared from the protected nonapeptide previously used for the synthesis of oxytocin(1), namely S-benzyl-N - carbobenzoxy - L - cysteinyl - L - tyrosyl-L - isoleucyl - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. For the present experiments, this protected nonapeptide was prepared by the stepwise synthesis recently described by Bodanszky and du Vigneaud(2). To obtain the protected decapeptide, the carbobenzoxy group was first removed from the protected nonapeptide by treatment with HBr in acetic acid and the resulting hydrobromide of the S,S'-dibenzyl derivative of reduced oxytocin

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