the antibody excess region. It can be seen that the whey contains a component identical with serum albumin and that this component constitutes near 2.5% of the whey proteins The immunochemical reactions with antibody to γ_2-globulin gave assay values from an initial 2.5% to values near 1%. The data indicate that a cross-reactive protein(s) is present in the milk whey. It is known that γ_2-globulins show immunological cross-reactions with other proteins in the γ_1- and β-globulin areas(14-16) and the present results may indicate the presence of other serum proteins in the milk whey.

Summary. The results of electrophoretic, ultracentrifugal and immunochemical analyses indicate that serum albumin is present in human milk whey. The immunochemical assay shows that it comprises 2.5% of this system. Proteins with the physical properties of human γ_2-globulin could not be demonstrated although material showing immunological cross-reactions with such proteins is present.

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Inactivation of Biologically Active ("Endotoxic") Polysaccharides by Fresh Human Serum. (23596)

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Recent work in this laboratory concerned with the interaction of endotoxic polysaccharides with biological systems has utilized inactivation of T2r+ coli phage as an indicator for the reaction with properdin. As expected, such polysaccharides nullified the anti-viral effect of human serum properdin. The influence of serum on other biologic activities of these polysaccharides was next investigated. The first *in vivo* phenomenon examined was the response of mouse sarcoma to a tumor-necrotizing polysaccharide after incubation with serum of known properdin content. When it was found that fresh serum abolished this property, it was first thought that this was a consequence of complexing with properdin. Contrary to expectation, however, incubation with properdin-deficient serum also inactivated the polysaccharide. These findings brought to mind unpublished experiments (1955) designed by Pillemer and Landy to detect physicochemical changes in *Salmonella typhosa* endotoxin upon exposure to fresh human serum. This treatment greatly increased the dispersion of the polysaccharide: the turbidity markedly de-

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TABLE I. Inactivation of Tumor-Damaging Potency of Polysaccharides with Fresh Human Serum.

Polysaccharide		Damage induced* in S37 after prior incubation of polysaccharide with:	
Source	Dose,† μg/mouse	Saline	Fresh serum
Ser. marcescens	10	9/10	0/10
S. typhosa	3	8/10	0/10
Sarcoma 37	50	10/10	2/10
Erythrocytes	40	8/10	0/10
Hellebore	30	5/10	0/10

* $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of treated mice}}$.

† Contained in 0.4 ml of saline or of fresh serum, after incubation for 1 hr at 37°C.

creased; it no longer sedimented at low speed; and it could not be centrifuged down even at high speed.† This change was also effected by RP and R3 sera. It was therefore evident to these investigators that this unsuspected attribute of serum was independent of properdin. This finding thus indicated the presence in serum of a factor, other than properdin, which is capable of modifying endotoxin. The present report deals with the biologic properties of selected polysaccharides as affected by incubation with serum.

Methods. Five polysaccharides were derived(2) from *Serratia marcescens, S. typhosa,* mouse Sarcoma 37, human erythrocytes, and a plant (Green Hellebore). Changes in potency were measured(3) chiefly with regard to induction of hemorrhagic necrosis in mouse Sarcoma 37, but also with regard to production of lethal shock in rabbits, the local Shwartzman reaction, and dermal necrosis in epinephrine-treated skin. An amount of polysaccharide, in saline, just sufficient to induce hemorrhagic necrosis in tumors was added to 0.4 ml of human serum and incubated for one hour at 37°C.

Results. Repeated experiments showed that fresh serum abolished the tumor-damaging potency of polysaccharides (Table I). However, serum heated at 56°C for 30 minutes prior to incubation, failed to inactivate the *Ser. marcescens* polysaccharide.

In the expectation that properdin might be involved, a properdin-deficient aliquot of the same human serum was prepared; to our surprise, the serum still inactivated the polysaccharide, thus indicating that properdin was not responsible for this effect. This inference was strengthened by obtaining similar results with serum completely lacking in properdin activity (prepared by addition of rabbit antiserum to human properdin). Moreover, aliquots of a serum which were made deficient, separately, in each of the components of complement and in Mg ions, all retained the ability to annul the tumor-necrotizing potency of the polysaccharides.

Lengthened incubation time increased the amount of polysaccharide counteracted by fresh serum. At 10°C no neutralization was obtained, even with incubation for 4 hours. Inactivation was the same at pH 5.8 and 7.8. KCN (1 mM) did not block the action of fresh serum, but the same concentration of $HgCl_2$ or of As_2O_3 did; as expected, cysteine (0.1 M) prevented the blocking by As_2O_3. No one of the chemical compounds, at these dose levels, damaged tumors.

In contrast to human serum, fresh serum from a rabbit failed to inactivate the polysaccharide from *Ser. marcescens.* However, serum from the same rabbit did inactivate it after the animal had been made tolerant by repeated injection of typhoid endotoxin. Fresh serum from mice had only a slight counteracting effect; that from the guinea pig was quite active. In this report human serum was used unless otherwise stated.

Continuous intravenous administration of certain polysaccharides to rabbits resulted in progressive leucopenia, hypothermia, cyanosis, respiratory difficulty and, frequently, terminal collapse(4). This sequence of "endotoxic" effects was abolished upon incubating *S. typhosa* polysaccharide with fresh serum for one hour at 37°C prior to administration by intravenous drip into rabbits. On the following day, the same animals received, by i.v. drip, an identical amount of the polysaccharide which had been incubated either with heat-inactivated serum or with

† The redistribution of endotoxin between sediment and supernatant solution was measured by 2 immunological technics in collaboration with A. G. Johnson and R-J. Trapani. Subsequently Stauch and Johnson(1) extended these observations.

saline; the typical endotoxic syndrome thereupon appeared, terminating in death. Immediate autopsy of the rabbits revealed no evidence of the renal cortical necrosis characteristic of the generalized Shwartzman reaction. If, however, the second i.v. treatment was performed with polysaccharide incubated in fresh serum, rather than in heat-inactivated serum or in saline, the endotoxic manifestations were not observed.

Fresh serum also annulled polysaccharide potency in the preparation of rabbit skin for the local Shwartzman reaction. Appropriate amounts of the various polysaccharides were incubated at 37°C for one hour with fresh serum, heat-inactivated serum, or saline. Equal amounts of these 3 reaction mixtures were injected into the abdominal skin of the same rabbits. Eighteen hours later the animals received the provocative dose of polysaccharide intravenously. The results 6 to 24 hours later showed that the fresh serum had abolished the preparatory activity of the polysaccharide whereas neither heat-inactivated serum nor saline diminished it.

It has already been shown(4) that these polysaccharides act as preparatory agents for the evocation of dermal hemorrhagic necrosis by epinephrine. After incubation for one hour at 37°C with fresh or heated serum, or with saline, polysaccharides were given i.v. to rabbits. One hour later the animals received intradermal injections of epinephrine in shaved abdominal areas. The local skin reactions were recorded after 6 and 24 hours. As was found in other phenomena, only incubation with fresh serum eliminated the effect of the polysaccharides.

Discussion. Several quite recent publications have reported on the action of fresh serum upon bacterial polysaccharides. Hegemann found that fresh human serum neutralized the pyrogenicity of crude culture filtrates from Gram negative bacteria(5); that it also neutralized purified lipopolysaccharides of these bacteria(6); and that prior heating of serum to 57°C for 45 minutes diminished its polysaccharide-neutralizing property(7). This loss of pyrogenic potency required incubation for 5 to 8 hours whereas in our experiments one hour incubation sufficed. Rowley reported that fresh rat serum split phosphate from a bacterial lipopolysaccharide (8), and that this serum activity was heat labile, appeared to require divalent ions, and exhibited a sharp pH optimum. However, no information was given on alteration of the biologic activities of the lipopolysaccharide. Ho and Kass have reported that human plasma partially protected rats against the lethal action of crude endotoxin(9), but this plasma factor was heat stable. Stauch and Johnson(1) reported on the alteration of specific precipitability of typhoid endotoxin after incubation with serum; this serum action presumably was heat stable and required incubation for 4 hours for maximum effect. Rall, Kelly and co-workers(10,11) found that fresh rabbit serum reduced, slightly, the pyrogenic activity of a bacterial polysaccharide. When rabbits were made tolerant to this polysaccharide, a greater reduction in pyrogenicity resulted. They also found that fresh rabbit serum partially protected mice against the lethal effect of a crude polysaccharide preparation, but did not protect against the tumor-necrotizing property. These diverse findings on the modifying effect of fresh serum on endotoxins refer to factors which in most instances differ from the one described in the present report, *e.g.*, one had a sharp pH maximum, and another was heat stable. Details of the present study will be reported separately.

Summary. Insofar as they have been examined, all the endotoxic properties of polysaccharides, irrespective of their source (*viz.*, bacterial, plant or mammalian), were annulled on incubation with fresh human serum but not with heated serum. Moreover, this action of fresh serum was independent of antibody, of complement, and of properdin.

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Acetylcholine-Cholinesterase Relationships in Embryonic Chick Lung Cultivated *in vitro*.* (23597)

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Acetylcholine has been shown to influence the level of cholinesterase activity of embryonic chick intestine cultured *in vitro*(1,2). Jones *et al.*(1) suggested that acetylcholine induced the formation of cholinesterase in intestinal cells, but since the added ester served only to prevent a consistent fall in cholinesterase activity during the culture period, no definite statement as to whether acetylcholine actually induced the formation of new cholinesterase could be made. The present report deals with the influence of acetylcholine and similar esters on the cholinesterase activity of embryonic chick lung, a tissue chosen because the authors hoped its normally relatively low level of cholinesterase activity would provide a setting wherein the formation of new cholinesterase following a substrate stimulus could be demonstrated. Lung cells served well for this purpose, and the system was used to study the relationship between enzyme induction and time of exposure to substrate, as well as the type of cholinesterase induced.

Methods. Primary explants of 15-day chick embryo lung were incubated at 37°C for 8 days in roller tubes in 25% embryo extract in a highly buffered salt solution according to methods described by Jones *et al.* (1). Acetylcholine and other compounds were added to the medium in amount sufficient to provide an initial concentration of 0.02 Molar. The *cholinesterase* activity and protein contents of the cells in individual roller tubes were measured by the microchemical methods described by Bonting and Featherstone(3). In experiments comparing the hydrolysis of several concentrations of various substrates, the cells of many roller tubes were pooled and homogenized, and enzyme activity was determined manometrically. Acetylcholine chloride and acetyl-β-methylcholine chloride were purchased from Merck and Company; propionylcholine iodide and butyrylcholine iodide were purchased from Dajac Laboratories at Leominster, Mass., and tributyrin was purchased from Fisher Scientific Co. Benzoylcholine was a gift from Hoffmann-LaRoche.

Results. The effects of adding 0.02 M acetylcholine or the products of its hydrolysis to the medium of embryonic chick lung cultivated *in vitro* for 8 days are shown in Table I for 2 experiments—one in which there was good growth (protein increase) of the lung cells during incubation, the other where there was no such growth. A 2 to 6-fold increase in the cholinesterase activity over the non-incubated controls occurred. If the assumption is made that cholinesterase concentration is proportional to cholinesterase activity, then these results strongly support the hypothesis that acetylcholine induced the formation of the cholinesterase. That acetyl-

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