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OBSERVATIONS ON A SUBSTANCE IN HUMAN PLASMA WHICH GIVES A SLOW CONTRACTION OF GUINEA-PIG GUT *IN VITRO*

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It has been known for many years that serum and plasma stimulate smooth muscle. This property of serum has been recently attributed to 5-hydroxytryptamine (Rapport, 1949). The vasoconstrictor and muscle stimulating properties of plasma and of serum were attributed by Stewart and Zucker (1913) to a change that takes place in shed blood. These properties are not developed —at least not fully—with the change of fibrinogen to fibrin, since the constrictor action of different plasmas (citrate, peptone or hirudin plasma) differs greatly, whereas the absence of coagulation is common to all.

The G2 fraction of human plasma causes a slow contraction of the isolated guinea-pig gut *in vitro*. The same effect has been observed with human blood plasma. Previous investigators (O'Connor, 1912; Trendelenburg, 1911; and Kahn, 1912) showed that the smooth muscle stimulating property of serum is, in general, greater than that of plasma. If this effect, as has been suggested, arises from preliminary stages in coagulation, or breakdown of platelets, it may still occur in non-coagulable blood, for the coagulation process may be interrupted at different points. The prevention of clotting has little influence on the development of the smooth muscle stimulating property. Coagulation of blood, from which the G2 fraction has been obtained, can be prevented by the addition of trisodium citrate. As the cellular elements then rapidly disintegrate it is probable that the preliminary stages of coagulation occur. There is, therefore, no reason to believe that the slow muscle contractor substance (SCS) in the G2 fraction from plasma will be chemically different from that in serum.

This paper describes the isolation and chemical nature of SCS from the G2 fraction of human blood plasma. A structure is proposed for a fatty acid which was isolated and which may be responsible for the effect on guinea-pig jejunum.

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METHODS

Chemical

Preparation of the G2 Fraction from Human Blood Plasma.—The G2 fraction was precipitated from human blood plasma by the ether method of Kekwick and Mackay (1948) after the separation of fibrinogen and prothrombin.

Isolation of SCS from the G2 Fraction.—The methods of purification involved extractions with organic solvents or redistilled water only. The alumina (Savory and Moore) used for chromatographic analysis of SCS yielded some inorganic impurities when aqueous methanol was the solvent, but these were insoluble in absolute methanol and redistilled ether, which were therefore used for the further purification of the active material.

The isolation consisted of five steps :

Step 1. *Extraction with Aqueous Ethanol.*—The G2 material (dry wt. 120 g.) precipitated from 13.5 l. of human plasma was suspended in 0.85% saline (1.2 l.), and N-sodium hydroxide added dropwise to *p*H 7.3. After the addition of 1.8 l. of 96% ethanol, the mixture was thoroughly stirred and left at room temperature overnight. The protein precipitate was removed by centrifugation and filtration and the filtrate evaporated below 40° C. and under reduced pressure to give 20–30 ml. of a fatty emulsion. Octan-2-ol was added to control foaming. The dried extract weighed 7.2 g., 100 units/g. (one unit is the activity of 50 mg. G2; see Table I).

Step 2. *Removal of Material Insoluble in Acetone.* —To the concentrate thus obtained, 10 vol. acetone were added. The mixture was shaken, stirred at room temperature for 30 min. and kept at 1–2° C. overnight. The insoluble material (dry wt. 4.2 g., no activity detected) was removed and the clear, light yellow supernatant solution evaporated to dryness under reduced pressure and below 40° C. The wet residue was partly dehydrated by recovering it from solution in butanol, by distillation *in vacuo* and by storing over P_2O_5 *in vacuo*. The dry solid (dry wt. 4.8 g., 200

units/g.) was dissolved in about 2 ml. of absolute methanol containing 10% anhydrous acetone; any insoluble material which formed was removed by centrifugation.

Step 3. *Chromatography.*—The material obtained in step 2 was applied to a column (Savory and Moore) of alumina (25 × 1.5 cm.) previously washed with 100 ml. absolute methanol containing 10% acetone, and developed with 200 ml. of the same solvent mixture. This eluate when dried had an activity of 1,000 units/g. The active substance was eluted from the column by 50% aqueous methanol, the first 80 ml. of the eluate being discarded. The following 150 ml. of effluent was evaporated to dryness under reduced pressure and the solid obtained was extracted with a few ml. absolute methanol. About 50 mg. active material (20,000 units/g.) was obtained.

Step 4. *Acetic Acid Precipitation.*—The material (step 3) was dissolved in 5 ml. warm distilled water. Glacial acetic acid (AR, 0.1 ml.) was added with stirring, and the copious white precipitate that formed was left in the cold for 1 hr. to form a coherent mat. The insoluble material was separated by filtration and

TABLE I

THE CONCENTRATION OF SCS IN, AND ITS ISOLATION FROM, THE G2 FRACTION OF HUMAN PLASMA

Fraction	Recovery of SCS from G2 (U./g. G2)	U.*/g. Dry Weight
G2	20	20
60% alcohol extract	12	100
Acetone extract ..	9	200
50% methanol eluate	8	20,000
Acid precipitate ..	5	20,000
Crystalline substance	5	50,000

* One unit of SCS is defined as that quantity which causes, in the isolated guinea-pig jejunum suspended in a 10 ml. bath, a slow contraction similar to that obtained with 50 mg. of G2.

centrifugation, washed with 1 ml. portions of 2% acetic acid, and finally removed from the filter paper with 5 ml. hot absolute methanol. A yellowish, transparent, semi-crystalline substance was obtained (28 mg., 20,000 units/g.).

Step 5. *Ether Extraction.*—The acid precipitate (step 4) was dried over P_2O_5 and NaOH pellets and the material was extracted with 5 ml. peroxide-free ether. The colourless crystalline material obtained from the ether solution was then further purified by extraction with acetone in the cold (10% solution at −10° C.). The acetone soluble material was crystallized from peroxide-free ether to yield about 12 mg. of a colourless crystalline substance (m.p. 34° C., 50,000 units/g.).

This method of isolation resulted in more than a 1,000-fold concentration of the substance on a dry weight basis (Table I). The recovery of activity is about 25% of that of the original G2 fraction.

Biological Assays

Rabbit Jejunum.—The determinations on isolated rabbit jejunum were made in a Dale's apparatus in the usual way, using a chamber of 10 ml. capacity. Fig. 1 shows the effect of atropine on acetylcholine and G2 fraction responses. The G2 fraction or acetylcholine was injected into the organ bath 3 min. after atropine. With acetylcholine, the jejunum was washed directly after the contraction had reached its summit. With the G2 fraction the jejunum was washed 1 min. after injection. Note the slow relaxation of the jejunum, which took 5–8 min.

Guinea-pig Jejunum.—The determinations on isolated guinea-pig jejunum were made in a Dale apparatus in the usual way, using a chamber of 10 ml. capacity. Solutions of the unknown were diluted until 0.2 ml. gave a response identical with that of 0.2 ml. of a standard control solution of SCS. The standard was a solution of G2 fraction pooled from 5 runs. A concentration of 250 mg./ml. was arbitrarily designated as containing 1 unit of activity. Fig. 2 shows the effect of G acid (the crystalline SCS) on isolated guinea-pig jejunum. Note the slow contraction of the jejunum, which started 20–30 sec. after adding G acid to the organ bath. When the contraction reached its maximum 1 min. after injection, the organ bath was washed out. The contraction persisted after washing out the organ bath, slowly returning to the base line. This took 2–3 min.

Rabbit Coronaries.—The isolated rabbit heart was perfused via the aorta with Ringer Locke solution, the perfusate being collected and measured. The decrease in the rate of flow following injection of G2 fraction or G acid was taken as a measure of coronary constriction. The heart has not been used as a routine test.

Haemolysis.—The methods used to assay the haemolytic activity of G acid were essentially those used by Laser (1949) with vaccenic acid. Human erythrocytes obtained from defibrinated blood were used. A few ml. of erythrocytes were twice washed with 0.15 M-phosphate buffer (*p*H 7.3) and 5–10% (v/v) suspensions prepared. A given suspension was divided into several parts and a fresh one was used for each series to prevent protracted incubation at 37° C. before zero time. All experiments were done with 15 ml. test-tubes in a hot room at 37° C. The tubes were placed in a metal stand and viewed against a light source. End-points of haemolysis were reached when all cloudiness in the test-tubes had disappeared. The suspension medium was 0.15 M-phosphate buffer from Na_2HPO_4 and KH_2PO_4. A known amount of G acid was dissolved by warming in 0.32 N-NaOH. Stock solutions containing 0.5–1.0 mg./ml. were prepared. G acid and the erythrocyte suspension were separately incubated in test-tubes until they reached 37° C. G acid was then added to the test-tubes containing the medium, the erythrocytes being added last, at zero time. The contents of each test-tube were mixed by inverting the tube. To prevent a *p*H shift of the medium used for the measurement of haemolysis, 0.1 ml. 0.32 N-HCl was added for each 100 mg. of G acid dissolved in NaOH.

RESULTS

Chemical

Some Physical and Chemical Aspects of SCS.—The small amounts of SCS available prevented a detailed study of its physical and chemical properties. The isolation of derivatives or decomposition products was not attempted. However, ultra-violet absorption spectroscopy and paper chromatography helped to establish the purity and chemical nature of the substance.

Pure SCS oxidizes, and becomes inactive, easily. The material in the G2 fraction of plasma is probably protected by a stabilizer. A G2 fraction in saline kept at 1° C. for six months retained its activity unchanged, whereas a solution of the pure SCS can lose over 90% of its activity after one month under the same conditions.

The SCS was strongly acid, and the neutral equivalent, determined by the method of Fairbairn (1945), was 283. Since oxidation with potassium permanganate in acetone gave only one dicarboxylic acid (malonic acid), this value of 283 is considered as the molecular weight of a fatty acid. SCS will therefore subsequently be referred to as G acid. The iodine number of G acid, determined by the micro-method of Yasuda (1931), is 75. This relatively low value suggests that the double bond is close to the carboxyl group (theoretical 89.7 for one double bond in a C_{18} fatty acid). Freshly isolated G acid was used for chemical analysis, since old specimens give higher figures for the neutral equivalent value (theoretical 282) and low figures for the iodine value. This behaviour may be attributed to partial oxidation or polymerization of G acid. These changes were, however, suppressed by keeping the G acid in ampoules sealed in nitrogen.

Examination of surface film formed by this acid on a Langmuir trough suggested that: (a) It is a straight chain acid because the film compressed to 20 Å^2/molecule. Stenhagen (1940) has shown that disubstituted acetic acids occupy areas greater than 20 Å^2 (α-methyl, 29.5 Å^2). This film was on 0.01 N-HCl. (b) The film was also spread over 0.01 N-sulphuric acid containing 0.0025 N-permanganate. An expansion of the film resulted, and on compression the film behaved as if it were partly dissolving, which is what oleic acid does under similar conditions.

G acid has a characteristic ultra-violet absorption in the region 2,200–2,250 Å. The value of $E^{1\%}_{1\,cm.}$ in this region is 34.7. Similar ultra-violet absorption has been found for 2-octadecenoic acid in which the ethylenic bond is conjugated with the carbonyl double bond in the carboxyl group (Myers, 1951). In this instance, however, the amount of absorption is greater.

The amount of active hydrogen in G acid is 0.35% as determined by Drs. G. Weiler and F. B. Strauss. This value is compatible with the presence of one replaceable hydrogen atom in the molecule, that is, the carboxyl hydrogen atom. Presumably, no hydroxyl group is present. No keto groups could be identified, since no phenyl hydrazone derivative could be isolated. The probability of an epoxy mode of combination is rather remote, since no reaction took place with ammonia under pressure, and no amino acid was detected after the reaction (Swern and Findlay, 1952).

Oxidation of G acid with potassium permanganate in acetone gave one dicarboxylic acid, which moved on paper using an ethanol-ammonia-water mixture for irrigation at the same rate as an authentic sample of malonic acid (Long, Quale and Stedman, 1951), and a monocarboxylic acid which was indistinguishable on paper from pentadecylenic acid. It is probable, therefore, that the double bond in G acid occupies the 3–4 position in a C_{18} straight chain.

The criteria given below, together with the properties already described, lead one to believe that the isolated G acid is not grossly contaminated with other substances.

1. The absence of nitrogen eliminates the presence of bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949).

2. The failure to get biologically or chemically different fractions from the crystalline G acid by crystallization from acetone at low temperatures (Brown, 1941) suggests the absence of appreciable amounts of saturated fatty acids as contaminants.

3. The absence of higher unsaturated fatty acids (linoleic, linolenic and arachidonic acids) was indicated, since no distinct increase in ultra-violet absorption was detected in the regions 2,350 Å, 2,700 Å and 3,000 Å (Wiese and Hansen, 1953), after treatment of the G acid with alkali by the method of Hilditch, Morton, and Riley (1945).

4. The demonstration on paper of only one dicarboxylic acid after cleavage of the double bond with potassium permanganate in acetone solution excludes significant contamination by other isomeric unsaturated fatty acids.

Biological

Action on Guinea-pig Jejunum.—Guinea-pig jejunum is stimulated by the G2 fraction in a manner unlike that of histamine or acetylcholine. The G2 fraction, after a latent period of about 20 or 30 sec., produced a contraction which did not

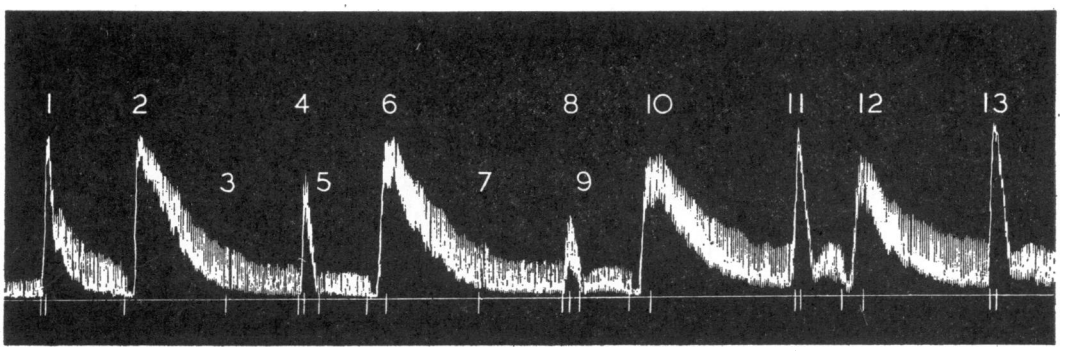

FIG. 1.—Rabbit jejunum. The effect of atropine on acetylcholine (ACh) and G2 fraction responses. (1) 1 μg. ACh. (2) 50 mg. G2 fraction. (3) 0.04 μg. atropine sulphate. (4) 1 μg. ACh. (5) 0.04 μg. atropine sulphate. (6) 50 mg. G2 fraction. (7) 0.1 μg. atropine sulphate. (8) 1 μg. ACh. (9) 0.1 μg. atropine sulphate. (10) 50 mg. G2 fraction. (11) 1 μg. ACh. (12) 50 mg. G2 fraction. (13) 1 μg. ACh.

reach its maximum for 1–2 min. Longer or shorter latent periods were sometimes observed, however, and, indeed, totally unresponsive preparations were occasionally encountered. Other characteristic features were that the contraction persisted after washing out the bath and that relaxation was slow. The effect of G2 fraction on smooth muscle was not antagonized by antazoline or by atropine. Rabbit jejunum behaved in a similar manner (Fig. 1).

The G acid produced a slow contraction similar to the G2 fraction. In a 10 ml. bath, 50 μg. G acid gave a response similar to 50 mg. G2 fraction on this preparation except that with the former the latent period was less (Fig. 2).

Other smooth muscle preparations have been used from time to time to investigate the biological aspects of the problem. The response of the isolated rabbit jejunum to the G2 fraction suggests that the active principle is not histamine. G2 fraction had no effect on isolated rat colon. This result rules out the presence of appreciable amounts of many pharmacological substances (for example, acetylcholine or 5-hydroxytryptamine).

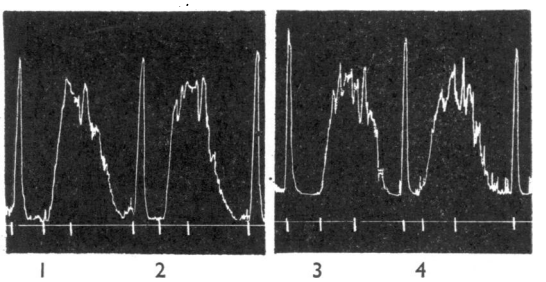

FIG. 2.—The effect of G acid on isolated guinea-pig jejunum. (1) 0.1 mg. G acid. (2) 0.1 mg. G acid. (3) 50 μg. G acid. (4) 50 μg. G acid. Other contractions are due to 0.2 μg. acetylcholine.

Rabbit Coronary Arteries.—0.2 mg. of G acid gave a definite but transitory constriction of the coronary arteries similar to that obtained with 1–2 mg. G2 fraction. The pure substance also produced a moderate and persistent decrease in the amplitude of the heart beat. This also occurred with the G2 fraction, but it was neither

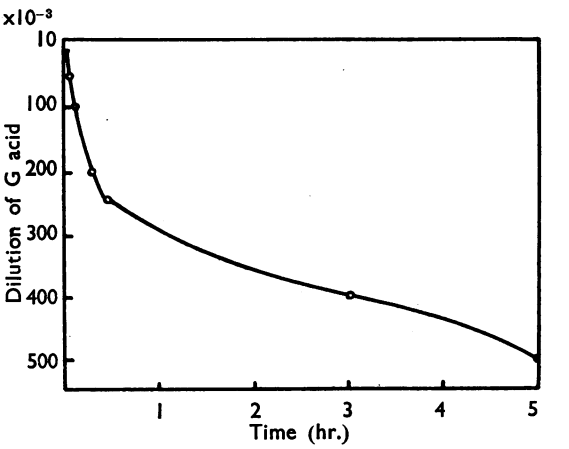

FIG. 3.—Relation between dilution of G acid and rate of haemolysis; 10 ml. phosphate buffer *p*H 7.3; 0.1 ml. 5% erythrocyte suspension.

so definite nor so persistent. These results suggest that some other substance is responsible for most of the constriction of rabbit coronary arteries. The contribution of G acid to the total coronary constrictor activity of the G2 fraction must be very small, for the G acid is 1,000 times more potent than the G2 fraction in producing slow muscular contraction, whereas it is only 7.5 times more potent as a coronary constrictor.

Haemolytic Activity.—Slow muscle-stimulating and coronary-constricting activities are present in

the original G2 fraction, whereas the haemolytic activity of the G acid is completely masked. Fig. 3 shows the relation between dilution of G acid and rate of haemolysis.

Effect of Calcium Ions.—To 5 ml. of 0.1% solution of G acid (sodium salt) in saline, an equivalent amount of calcium chloride in 0.1 ml. water was added. The solution was stirred, allowed to stand at 1° C. for 1 hr., and then filtered. The filtrate was assayed on the isolated guinea-pig ileum and human red cells. The haemolytic activity was completely lost, and the slow contractor activity was reduced by about 90%.

The residual 10% of the slow contractor activity manifested by the so-called pure substance in the presence of an equivalent amount of calcium ions in a protein free system may be attributed to the presence of another substance whose action on the isolated guinea-pig ileum is not affected by calcium ions, or to the possible partial dissociation of the calcium soap formed by G acid at the relatively high dilution of the biological assay. In a 10 ml. Ringer's organ bath where an isolated piece of guinea-pig ileum shows its maximum response to 50 μg. of pure G acid, the amount of calcium chloride present is about 250 times more than the equivalent amount necessary to inhibit 90% of the slow contractor activity of 50 μg. of G acid in a protein free concentrated solution (1 mg. of G acid/ml.). However, calcium ions do not seem to interfere with the slow contractor activity of G acid under the conditions of biological assay. These considerations indicate that the inhibiting effect of calcium ions upon the slow contractor activity of G acid is chemical and not physiological. The same considerations make it obvious that if G acid is present in plasma its slow contractor activity would not be affected by calcium ions, since their concentration in human blood serum (about 5 mg./100 ml. under normal conditions) is less than that present in the Ringer solution used (8.7 mg./100 ml.).

Discussion

Some slow muscle contracting substances seem to arise through the action of proteolytic and lipolytic enzymes on certain precursors. For example, bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin, has been described by Rocha e Silva *et al.* (1949). It has also been reported (Braun-Menéndez *et al.*, 1943) that the action of renin on the pseudo-globulin fraction of normal plasma or serum produces two substances with smooth muscle stimulating actions, one of which is hypertensive (hypertensin) and the other hypotensive.

In 1938 Feldberg and Kellaway, in experiments on perfused lungs, found that the venous perfusate after injection of snake venom caused not only a rapid histamine-like contraction but also a delayed and slow contraction of isolated guinea-pig jejunum. According to these two authors, its formation probably accounted for the stimulating action of the venom on the gut. Also, lymph and egg yolk treated with venom acquired a lytic action on the red blood cells and caused contraction of the isolated jejunum, which had been desensitized to venom. Feldberg and Kellaway thought that these activities were properties of at least two substances, the haemolytic activity being attributed to lysolecithin.

The chemical nature of the slow contractor substance isolated from human plasma suggests that the slow contraction described by Feldberg and Kellaway in their experiments with venom-perfused lungs may have been due to an unsaturated fatty acid identical with, or similar to, G acid. The splitting of this from the parent phospholipid molecule is effected by lecithinase A present in snake venom. When one considers the slow contractor action of G acid in relation to known fatty acids, the following considerations emerge.

Among the saturated fatty acids, those between C_2 and C_{18} were inactive in a dose of 200 μg. The monoethenoid unsaturated C_{18} fatty acids *cis*-9-octadecenoic, *trans*-9-octadecenoic and *cis*-6-octadecenoic acids were also inactive at the same dose. *cis*-11-Octadecenoic acid (*cis*-vaccenic acid), on the other hand, has much the same effect in similar doses as G acid on the isolated guinea-pig jejunum and on rabbit coronary arteries. However, according to the work of Laser (1949) it appears that the haemolytic activity of *cis*-11-octadecenoic acid isolated from horse brain is nearly 3 times that of G acid isolated from the G2 fraction of human plasma. Moreover, a study of the physical and chemical properties of G acid suggests that it differs from *cis*-11-octadecenoic acid with respect to the double bond in the hydrocarbon chain of the fatty acid. The double bond in *cis*-vaccenic acid was proved by Morton and Todd (1950) to be in the 11–12 position. In G acid, however, it seems probable that the double bond occupies the 3–4 position. The diethenoid *cis*-9:12-octadecadienoic acid acts on the isolated guinea-pig jejunum in a dose of 50 μg. The type of contraction and recovery after washing is similar to that after G acid. A dose of 200 μg. of *cis*-9:12-

octadecadienoic acid, however, is practically inactive on rabbit coronaries. In a dose of 200 μg. the triethenoid *cis*-9:12:15-octadecatrienoic acid rendered the isolated guinea-pig jejunum unresponsive to various stimulating agents.

Thus, it may be expected that the slow contractor activity of a given unsaturated fatty acid depends on the number and position of the double bonds in the hydrocarbon chain. These haemolytic and slow contractor activities may be due to different structural areas of the fatty acid molecule. If this is so, then by using such inhibitors of haemolysis as plasma, albumin, or the starting G2 fraction, it should be possible to mask completely the haemolytic activity of the fatty acid in question, and leave the slow contractor activity unaffected. These conditions are fully satisfied with *cis*-9:12-octadecadienoic acid and G acid, which behave similarly.

Summary

1. A slow muscle contractor principle isolated from the G2 fraction of human plasma appears to be a long chain C_{18} unsaturated fatty acid. This principle has been called G acid.

2. It is possible that G acid is 3-octadecenoic acid.

3. G acid manifests the following three biological properties: (a) A slow and delayed contraction of the isolated jejunum of the guinea-pig in a concentration of 2×10^{-6}; (b) a marked haemolytic activity on human red cells in a concentration of 2×10^{-6}; and (c) a vasoconstrictor activity on rabbit coronary arteries in a dose of 0.2 mg. in the isolated perfused heart.

4. It seems probable that the centre of unsaturation in G acid is responsible for its biological properties.

5. The probable relation of G acid to the slow acting principles obtained by previous workers and to known fatty acids is discussed.

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