Chemistry and Biology of Lipids

XIII. The Group Lipoid of Pig Pancreas

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14 fresh pig pancreases were separately stood with cold alcohol and the dehydrated materials treated with hot alcohol. The substances extracted with cold alcohol, the ones, precipitating and non-precipitating from the hot-alcoholic extracts when cooled, and the tissue residues were examined regarding the blood group activity. The third fractions all specifically inhibited the isoagglutination of **A** erythrocytes, whereas, of the first fractions, some could be recognized to effect the same action but some not due to hemolysis. The other fractions proved inactive. Next, the balance of the third fractions were combined and subjected to the procedure in preparing the group lipoids of human pancreases¹⁾ with minor modifications. The divisions were tested on the sero-reactivity during the whole course, because between two kinds of animal, the corresponding substances of the same organs are not warranted to fall on the corresponding divisions in fractionation of the lipids in particular.

An electrophoretically homogeneous final product was much less active than the Group **A** lipoid of human pancreas and further diverged from the latter in composition to some extent.

EXPERIMENTAL

Examination of the Blood Group Activity of Pig Pancreases

14 fresh pig pancreases weighing from 42 to 115 g. were separately masticated and preserved in 3 volumes of 91% denatured alcohol. 5 of them were stood for 6 weeks and the remaining for 1 year. The materials were then collected on a funnel and after drying in a Faust apparatus, boiled twice with 5 volumes of the denatured alcohol for 2 hours. The successive extracts of each material, filtered while hot, were combined, placed in an ice chest for 1 day, whereby a precipitate (**II**) occurred. The

TABLE I
Weight of the Fractions of Pig Pancreases and Inhibition
of Isoagglutination of Erythrocytes

—: group-inactive, +: Group A active, ±: probable Group A activeness.

Pig pancreas		I* washed with acetone		Hot-alcoholic extract				Tissue residue after hot-alcoholic extraction	
No.	wt. (g.)	wt. (g.)	group-activity	II† washed with acetone		III‡ washed with acetone		wt. (g.)	group-activity
				wt. (g.)	group-activity	wt. (g.)	group-activity		
1	46	1.4	+	0.02	—	0.5	+	4.0	—
2	55	1.1	±	0.06	—	0.6	+	3.0	—
3	115	2.6	+	0.09	—	1.0	+	8.7	—
4	78	1.1	±	0.27	—	1.1	+	6.4	—
5	98	3.1	+	0.09	—	1.0	+	6.9	—
6	81			0.04	—	0.6	+	6.0	—
7	64			0.18		0.8	+	3.2	
8	94			0.09		0.9	+	6.2	
9	42			0.02		0.5	+	2.6	
10	92			0.18		0.8	+	7.7	
11	38			0.01		0.4	+	4.2	
12	71			0.09		0.6	+	2.9	
13	96			0.18		1.4	+	9.2	
14	59			0.05		0.3	+	2.1	

* The fraction extracted with cold alcohol. † The precipitate from the hot-alcoholic extract on cooling. ‡ The substance that had been recovered by distillation of the hot-alcoholic extract after cooling and removing of the precipitate.

TABLE II
Occurrence of Isohemoagglutination, when Fr.-I's,* washed
with Acetone, had been added to the Normal Anti-sera
The B and A serum had agglutinin titres of 320 and 80
and were diluted 20 and 5 times respectively.

—: no agglutination, ±: faint aggl., +: slight aggl., H: hemolysis.

Pig pancreas No.	Group of red cells	Dilution of substances				
		1:10 ²	1:2×10 ²	1:4×10 ²	1:8×10 ²	1:1.6×10 ³
1	A	H	H	H	H†, —	+
	B	H	H	H†, +	+	+
2	A	H	H	H	H†, ±	+
	B	H	H	H	H†, +	H†, +
3	A	H	H	H	H†, —	+
	B	H	H	H†, +	+	+
4	A	H	H	H	H	H†, ±
	B	H	H	H	H†, +	+
5	A	H	H	H	H†, —	H†, ±
	B	H	H	H	H†, +	+

* Signifies the same as in Table I. † Hemolysis was partial.

TABLE III

Occurrence of Isohemoagglutination, when Fr.-III's,* washed with Acetone, had been added to the Normal Anti-sera

The B and A serum had agglutinin titres of 320 and 80 and were diluted 20 and 5 times respectively.

—: no agglutination, $\pm$: faint aggl., +: slight aggl., ++: marked aggl., $\equiv$: complete aggl.

Pig pancreas No.	Group of red cells	Dilution of substances								
		1:10 ²	1:2 $\times$ 10 ²	1:4 $\times$ 10 ²	1:5 $\times$ 10 ²	1:8 $\times$ 10 ²	1:10 ³	1:1.6 $\times$ 10 ³	1:5 $\times$ 10 ³	1:10 ⁴
1	A	—			$\pm$		+		++	$\equiv$
	B	+			++		++		++	$\equiv$
2	A	—			—		+		++	$\equiv$
	B	+			++		++		++	$\equiv$
3	A	—			—		+		++	$\equiv$
	B	++			++		++		++	$\equiv$
4	A	—	—	—		$\pm$		+		
	B	+	+	+		+		+		
5	A	—	—	—		+		+		
	B	+	+	+		++		++		
6	A	—					+		++	$\equiv$
	B	++			++		++		++	$\equiv$
7	A	—	—	—		—		+		
	B	+	+	+		+		+		
8	A	—	—	—		—		+		
	B	+	+	++		++		++		
9	A	—	—	+		+		++		
	B	+	+	+		++		++		
10	A	—	—	—		+		+		
	B	+	+	+		++		++		
11	A	—	—	—		+		+		
	B	+	+	+		+		++		
12	A	—	—	—		—		+		
	B	+	+	+		+		++		
13	A	—	+	+		++		++		
	B	+	+	+		++		++		
14	A	—	—	—		—		+		
	B	+	+	+		+		+		

* Signifies the same as $\dagger$ in Table I.

centrifuged supernatant was distilled to dryness (**III**). The alcohol portions used for the preservation were treated likewise, and the still residues thereby (**I**'s) and the substances recovered from the hot alcoholic extracts were all washed with acetone and dried. **III**, washed with acetone, of all the pancreases were found to be specifically anti-isoagglutinative to **A** erythrocytes. **I**'s, washed with acetone, caused noticeable hemolysis so that definite judgement of the agglutination was allowed only in some of those tested, which indicated that the Group **A** lipoid had entered partially into the cold alcohol. The other fractions including the acetone-soluble and the tissue residues were inactive. A great part of the details of the experiment is embodied in Tables I, II and III.

Isolation of the Group Lipoid

1st stage. The balance of Fr-III's, washed with acetone, of the pig pancreases above were combined (10.5 g. altogether) and exhausted with three 150 cc. portions of ether (cold) and the solutions were distilled in vacuo and dried in a desiccator—Fr. **Sol_{et}** (a yellowish butterlike substance, 0.15 g.). It showed no inhibition of the isoagglutination of **A** and **B** erythrocytes at a dilution of 1:200 (Complete hemolysis occurred at 1:100 dilution of the substance). The insoluble part—Fr. **Ins_{et}** (a yellowish, non-sticky powder, 9.9 g.) was Group **A** active (inhibitory to isoagglutination of **A** erythrocytes at 1:500), but not reactive with β -agglutinin at 1:100. This was further divided, employing three 150 cc. portions of chloroform. The chloroform-soluble (Fr. **Sol_{chl}**), recovered from the solutions similar to above, amounted to 0.5 g. (a brownish, hard clump). When it was added, as dissolved in physiological saline, to the

Fig. 1. An electrophoretic pattern of a 0.86% solution of Fr. **Inschl** in phosphate buffer, pH 7.8, $I=0.29$; 5°C ; current 10 ma. Exposure 60 minutes after starting current. Ascending limb to the right (Boundaries were shifted before photographing).

TABLE IV

Occurrence of Isohemoagglutination, when Fractions at the First Stage of Preparation had been added to the Normal Anti-sera

The **B** and **A** serum had agglutinin titres of 320 and 80 and were diluted 20 and 5 times respectively.

—: no agglutination, +: slight aggl., ++: marked aggl., #: complete aggl., H: hemolysis.

Fraction	Group of red cells	Dilution of substances									
		1:10 ²	1:2×10 ²	1:4×10 ²	1:5×10 ²	1:8×10 ²	1:10 ³	1:1.6×10 ³	1:5×10 ³	1:10 ⁴	1:5×10 ⁴
Sol _{et} *	A	H	++	##		##		##			
	B	H	##	##		##		##			
Ins _{et} *	A	H			—		+		+	+	+
	B	+			+		++		##	##	##
Sol _{chl} *	A	H	±	+		+		++			
	B	+	+	++		++		##			
Inschl *	A	H			—		+		+	+	+
	B	+			++		++		++	++	++

* See the text.

normal antisera, **A** erythrocytes agglutinated only faintly at a dilution of 1:200 of it in the saline (hemolysis at 1:100) but **B** erythrocytes agglutinated markedly at its 1:100 dilution. The residue (Fr. **Ins_{chl}**)—a yellowish, hygroscopic, fine powder—had the same Group **A** potency as the ether-insoluble, and gave positive Molisch and biuret reaction, and electrophoresis of a 0.86% solution (somewhat turbid) in a buffer of pH 7.8 demonstrated two components (Fig. 1). This was mostly soluble in cold and hot methanol, cold neutral- and faintly-alkalinized-water, and hot acidulated water. Completely soluble in hot neutral- and faintly-alkalinized-water. Insoluble in ether, petrol ether and chloroform.

(Serological potencies of the fractions above are illustrated in detail in Table IV.)

2nd stage. It was kneaded with a little water and water added up to 300 cc., followed by vigorous agitation. The centrifugate was further exhausted with two 300 cc. portions of water. The clear yellowish supernatant were united (100 cc. altogether) and added to with 90 cc. of 5% lead acetate, and the centrifugate here washed with changes of a dilute solution of the salt with centrifugation. The supernatant were bubbled with H_2S . The black precipitate was separated and washed with hot water and the mother fluid and washings were distilled together to dryness and washed with acetone. 7.5 g. of a yellowish hygroscopic, fine powder (**Non-ppt_{Pb}**) were given which was group-inactive. The lead precipitate in 900 cc. of water was also treated with H_2S and the suspension was acidified to pH 1.0 with HCl. Centrifuged. The transparent yellowish supernatant was neutralized with NaOH, distilled and washed with acetone. And 0.6 g. of a brownish, very hygroscopic powder was given—**Ppt_{Pb}-Sol_{HCl}**. The centrifugate was washed with three 50 cc. portions of a very dilute HCl (pH 1.0) and then extracted repeatedly with methanol. In the second and following times, the extractant was added to with HCl to pH 6.0 to obtain a clear centrifuged supernatant. The solutions were concentrated by vacuum distillation, neutralizing halfway and rejecting some precipitate thereby, to dryness. The remainder was washed with acetone. 670 mg. of a non-sticky powder with light yellow-brown shade were given—**Ppt_{Pb}-Ins_{HCl}**. This product inhibited isoagglutination of **A** erythrocytes (complete inhibition at a dilution of 1:1000), but not that of **B** erythrocytes. Molisch positive, biuret negative. The electrophoresis revealed it was still heterogeneous. Here a 0.86% solution in an alkaline buffer was applied and two boundaries were demonstrated (Fig. 2). It was soluble partially in cold methanol, ethanol, benzene, tetralin and water, but mostly in hot methanol, ethanol and water and completely in hot tetralin and alkaline water. Insoluble was it in ether, petrol ether and chloroform.

TABLE V

Occurrence of Isohemoagglutination, when Fractions at the Second Stage of Preparation had been added to the Normal Anti-sera

The B and A serum had agglutinin titres of 320 and 80 and were diluted 20 and 5 times respectively.

—: no agglutination, +: slight aggl., ++: marked aggl.,
 ##: complete aggl., H: hemolysis.

Fraction	Group of red cells	Dilution of substances											
		1: 10 ²	1: 2 × 10 ²	1: 4 × 10 ²	1: 5 × 10 ²	1: 8 × 10 ²	1: 10 ³	1: 1.6 × 10 ³	1: 5 × 10 ³	1: 10 ⁴	1: 2 × 10 ⁴	1: 4 × 10 ⁴	
Non-pptPb *	A	##	##	##		##		##					
	B	##	##	##		##		##					
PptPb-SolHCl*	A	++	##	##		##		##					
	B	##	##	##		##		##					
PptPb-InsHCl*	A	—			—		—		+	+	+	##	
	B	H			+		++		++	++	##	##	

* See the text.

Fig. 2. An electrophoretic pattern of a 0.86% solution of PptPb-InsHCl in phosphate buffer, pH 7.8, I=0.29 (The substance was dissolved in the buffer by warming at 30°C for 2 minutes.); 5°C; 10 ma. Exposure 40 minutes after starting current. Ascending limb to the left.

3rd stage. 0.3 g. of it was subjected to the procedure of counter current distribution, employing 10 funnels. Solvent pair, an equilibrated mixture of methanol, benzene, toluene and water (4:4:1:1). The substance was at first dissolved in 150 cc. of the lower phase and distributed between that solvent and an equal volume of the upper phase. After the last transfer the content of all the funnels were distilled separately and the residuals washed with acetone and dried.

	Funnel No.									
	0	1	2	3	4	5	6	7	8	9
Weight of the products (mg.)	6	6	11	15	15	17	15	15	20	130

Their anti-isohemoagglutinative potencies are illustrated in Table VI, which shows the fraction from Funnel 9 only was definitely group (A)-active.

TABLE VI

Occurrence of Isohemoagglutination, when Fractions in the Third Stage of Preparation had been added to the Normal Anti-sera

The B and A serum had agglutinin titres of 320 and 80 and were diluted 20 and 5 times respectively.

—: no agglutination, ±: faint aggl., +: slight aggl., ++: marked aggl., #: complete aggl., H: hemolysis.

Fraction*	Group of red cells	Dilution of substances												
		1:10 ²	1:2 × 10 ²	1:4 × 10 ²	1:5 × 10 ²	1:8 × 10 ²	1:10 ³	1:1.6 × 10 ³	1:2 × 10 ³	1:3.2 × 10 ³	1:4 × 10 ³	1:6.4 × 10 ³	1:8 × 10 ³	1:1.2 × 10 ⁴
0	A B		+	+		+		±		±				
1	A B		+	+		+		+		+				
2	A B		±	±		±		#		#				
3	A B		+	+		+		+		+				
4	A B		+	+		+		+		+				
5	A B		+	+		+		+		+				
6	A B		+	+		+		+		+				
7	A B		H H	H +		H +		+		+				
8	A B				H H		H +		+	+	+	+	+	
9	A B				H +		H +		+	±	±	±	±	
9-Ins†	A B	H H	H H	H H		H H		H H		H H				
9-Sol†	A B	H H	H H	H +		H +		— +		± ±		+	+	+

* Figures express funnel numbers. † See the text.

In a similar run with the balance of the product in the foregoing stage (320 mg.), 140 mg. of a substance, washed with acetone, were recovered from Funnel 9.

The substances yielded in the both runs (265 mg. altogether) were shaken with 30 cc. of water on an apparatus for 6 hours and centrifuged. On the centrifugate was repeated the operation many times (300 cc. of water used in total). The insoluble part (a powder tinged yellowish) —**9-Ins**—amounted to 15 mg., and in the examination of the group

activity it caused complete hemolysis even at the dilution of 1:3,200 (Table VI). The solutions were distilled in vacuo and washed with acetone—240 mg. of a yellowish non-sticky powder (**9-Sol**) were given. This product was specifically Group **A** active. It also exhibited hemolytic action, though not strong, probably due to a trace of the water-insoluble part contaminating (Table VI). Electrophoretically it was homogeneous as is shown in Fig. 3.

Fig. 3. An electrophoretic pattern of a 0.67% solution of 9-Sol in phosphate buffer, pH 7.8, $I=0.29$; 11°C ; 15 ma. Exposure 60 minutes after starting current. Mobility $2.03\text{ cm}^2\cdot\text{volt}^{-1}\cdot\text{sec}^{-1}\cdot 10^5$. Ascending limb to the left.

Since, however, it contained an abundant amount of ash (64.4%, Pregl's method without use of H_2SO_4), 130 mg. of it were electrodialyzed as dissolved in 7 cc. of water (Pauli apparatus) until no shift of pH occurred in the outer fluids (2 days requisite). The substance precipitated thereby in part, but because of difficulty in separating the precipitate, the solution containing it was distilled as such in vacuo and dehydrated with acetone. A yellowish powder weighing 30 mg. was provided. Biuret, Goldschmiedt (for hexuronic acid), Neuberg & Saneyoshi (for hexuronic acid) and Rosenthaler (for methylpentose) negative; Molisch and Elson & Morgan positive. Composition, group-activity and solubilities are shown in Tables VII, VIII and IX.

TABLE VII
Composition of the Final Preparation of Pig Pancreas
Group Lipoid

Analysis	In per cent	In equiv. per equiv. wt.
N	4.2	2.9
P	3.2	1.0
Ferricyanide reduction of the hydrolystate* as glucose	9.2	
Ash†	3.5	

* The substance was hydrolyzed by 4 hours' heating with 1 N H_2SO_4 , as sealed in an ampoule. The hydrolysate contained none precipitable by phosphotungstic acid so that it was immediately neutralized for measurement of the reduction. † Pregl method without H_2SO_4 .

TABLE VIII

Occurrence of Isohemoagglutination, when the Final Preparation had been added to the Normal Anti-sera

The B and A serum had agglutinin titres of 320 and 80, and were diluted 20 and 5 times respectively.

—: no agglutination, +: slight aggl., ++: marked aggl.,
 ###: complete aggl., H: hemolysis.

Group of red cells	Dilution of the substance					
	1: 5 × 10 ²	1: 10 ³	1: 2 × 10 ³	1: 4 × 10 ³	1: 8 × 10 ³	1: 1.6 × 10 ⁴
A	H	H	—	+	+	+
B	+	+	+	+	++	##

TABLE IX

Solubilities of the Final Preparation

—: insoluble, ±: faintly sol., +: partly sol., ++: mostly sol.,
 ###: completely sol., cold=20°C, hot=50–60°C.

Solubilities	Solvents	Water						Me- thanol	Ethanol	Ether	Petrol ether	Ben- zene	Glacial acetic acid	Tetra- lin	Chloro- form			
		acid*		plain		alkaline†												
		cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	
±	— ~	±	— ~	+	+	~	±	+	~	±	±	—	+	+	±	±	—	—

* pH<1.2, † pH>9.6, ‡ A slightly opalescent solution formed.

Compared with the corresponding substance of human pancreas, the following remarks are given as to the present group lipid:—1) it is much less group-active; 2) the potency is not diminished by the third stage-operation; 3) the equivalent ratio of N and P of it (2.9:1.0) does not agree with that of the one in human pancreas (1.9:1.0); 4) it contains less carbohydrate and more N and P than the human substance.

SUMMARY

14 pig pancreases contained all a Group A lipid. The tissue residues after extraction with hot alcohol was group-inactive.

The group lipid was prepared in an electrophoretically homogeneous state. It analyzed: N 4.2%, P 3.2%, ferricyanide reduction of the hydrolysate as glucose 9.2%, ash 3.5%; and inhibited specifically the isoagglutination of A erythrocytes at a dilution of 1:2 × 10³. It dissolved