

THE PURIFICATION OF INSULIN AND SIMILAR SUBSTANCES BY SORPTION ON CHARCOAL AND SUBSEQUENT RECOVERY¹

P. J. MOLONEY, AND D. M. FINDLAY

"Since the adsorption of liquids by solids is selective it follows that one liquid will be adsorbed more by a solid than will a second liquid and that consequently the first will displace the second from contact with the solid. No systematic study of this phenomenon seems to have been made²." The same is true for the sorption of solutes³ or colloids from solution and it seems likely that systematic study of the latter cases would lead to valuable improvements in the technique of chemical purification. Many chemically ill-defined substances—dyes, toxins, etc.,—are readily removed from solution by shaking with charcoal or kaolin; if they could as readily be recovered from the sorber they would be accessible to chemical investigation.

Comparatively little experimental work has been published on the separation of sorbate from sorber; in connection with our endeavors to purify insulin by means of charcoal we have been led to classify the available methods into four main groups. Methods of the first group are applicable whether the sorption is reversible or not. Those of the other three only when it is at least approximately reversible.

GROUP I: *By chemical action on the sorber*, for instance:—

(A) by dissolving it, leaving the sorbate undissolved. Pepsin⁴ and lipase⁵ have been purified by sorption on cholestrin followed by solution of the latter in ether; similarly insulin⁶, by sorption on benzoic acid and subsequent treatment with ether.

(B) If a complex containing an amorphous sorber were kept under such conditions that crystallization set in, the sorbate would no doubt in many cases be released; Freundlich and Schucht⁷ showed that amorphous mercuric sulphide sorbs more dye than the crystalline form into which it spontaneously changes, but we have found no case of isolation or purification by this method.

¹ From the Insulin Division, Connaught Laboratories, University of Toronto.

² Baneroff: "Applied Colloid Chemistry", p. 72, (1921).

³ For the sake of brevity the word "solution" "solute", and "solvent" are employed to include colloidal suspensions, etc.; this avoids the continuous repetition of such terms of "solution or suspension", "solute or suspensoid", "solvent or dispersoid." The word "complex" is used as an abbreviation for "sorber plus sorbate."

⁴ E. Brücke: Virchow's Archiv. 25 279.

⁵ Willstätter: Hoppe-Seyler's Z. physiol. Chem. 125, 191 (1923).

⁶ J. Biol. Chem. 57, 359 (1923).

⁷ Z. physik. Chem. 85, 660 (1913).

(C) Finally if further study should show that the phosphate solutions used by Willstätter¹ convert alumina or kaolin into a phosphate incapable of sorbing enzymes, his method of purifying the latter would come into this group.

GROUP II: *By changing the temperature*, effecting the sorption at a temperature where the sorption coefficient is high and the removal at a temperature where it is low. This method has found wide application in the case of gases. Effront's² measurements of the effect of temperature on the sorption of ptyalin by filter paper might lead to a method of purifying the ptyalin if anything like equilibrium was reached in his experiments. This method is of general applicability and can be used in conjunction with those of the other groups.

GROUP III: *By lowering the chemical potential of the solute in the solution*. This may be effected:—

(A) by lowering its concentration, as by repeatedly washing the complex with fresh portions of the original solvent; e.g., the removal of sorbed sugar³ from charcoal by washing with water. Because of the general form of the sorption isotherm this method must be expected to give a poor yield and a very dilute solution of the product desired; it has been employed however by Effront,⁴ who purified pepsin by sorption on filter paper and washing out with water.

(B) by adding to the original solvent some fourth substance and thus in effect creating a new solvent in which the sorbate is more soluble than in that from which it is sorbed. Acids for instance, sorbed by wool⁵ from aqueous solution, may be recovered by digestion with dilute alkali; to recover the original acid of course requires a subsequent operation. Changing the pH of the solution from which a colloid has been sorbed might have the same effect. Ambard⁶ and Effront⁷ used dilute solutions of starch to remove amylase from starch granules and ptyalin from cellulose respectively, but did not recover the enzymes in the pure state.

(C) by the use of an entirely new solvent, e.g., iodine or methyl violet⁸ taken up by charcoal from aqueous solution may be recovered by treating the complex with alcohol.

GROUP IV: *By replacing the original sorbate by another*. This may be effected by digesting the complex with a solution, (or suspension) of some fourth substance which is readily sorbed and which does not itself carry the

¹ Hoppe-Seyler's Z. physiol. Chem.

² Compt. rend. Soc. Biol. **86**, 271.

³ Bayliss: "Principles of General Physiology" 2nd Ed. p. 69.

⁴ Compt. rend. Soc. Biol. **87**, 128.

⁵ Von Georgievics: Monatsh. f. Chem. **32**, 655 (1911).

⁶ Bull. Soc. Chim. Biol. **3**, 51-56.

⁷ Compt. rend. Soc. Biol. **86**, 271.

⁸ Bancroft: "Applied Colloid Chemistry, p. 113 (1921).

original sorbate into the interface.¹ Saponin² has been used to prevent rennet from concentrating in the foam of a shaken solution, and it has also been used to re-activate³ rennet sorbed on charcoal; Freundlich and Kaempffer⁴ observed that the sorption of uranium salts is decreased by the addition of thorium salts, benzoic acid, strychnine nitrate, etc., to the solution, and ascribed this to replacement of the uranium in the complex by these substances; but we have found no clear case of isolation and purification by replacement in the complex. However, since the boundary between groups I-C and IV is ill-defined, it may be that some Willstätter's⁵ work on the purification of enzymes comes under this head.

Although hitherto they have been but little used, we believe that the systematic application of the methods of the fourth group is the best means available for isolating and purifying the chemically ill-defined substances so important from the physiologist's point of view; it does not seem too much to hope that by their means a technique of practical sorption and recovery may be developed which will play the same part in the purification of colloids that fractional distillation and fractional crystallization play in the purification of volatile and crystalline chemicals.

Application to the Purification of Insulin

In our work charcoal is used as a sorber, about one milligram being needed to remove the insulin from 1 cc. of a relatively pure solution such as the clinical product.⁶

The methods of Group I are inapplicable with charcoal as a sorber; as stated above they gave us good results with benzoic acid. Method II was used in combination with the others; III-A (washing with water), proved useless. Under III-B come our attempts to recover the insulin by digesting the complex with aqueous solutions of varying pH—from hydrochloric acid of pH 1.0, to borate and phosphate buffers as alkaline as pH 9.8; negative results were obtained in every case, while greater alkalinity destroys the insulin. Experiments in which the complex was digested with various solvents⁷ of insulin—phenol, glacial acetic acid, or a mixture of hydrochloric acid and alcohol—come under III-C; the results here too were disappointing.

¹ Hunter (J. Chem. Soc. 23, 73 (1870)) has shown that the sorption of ammonia from gases by charcoal is increased by the presence of sorbed water.

² S. and S. Schmidt-Neilson: Hoppe-Seyler's Z. physiol. Chem. 68, 317 (1910)

³ Jahnsen-Blohm: Hoppe-Seyler's Z. physiol. Chem. 82, 178 (1912).

⁴ Z. physik. Chem. 90, 681 (1915).

⁵ Hoppe-Seyler's Z. physiol. Chem. 125, 132 (1923).

⁶ With a crude solution of insulin much larger amounts of charcoal are needed, due no doubt to the sorption of other constituents of the crude preparation. One might expect with Freundlich that constituents which lower the surface tension of the solution would be preferentially sorbed; this conclusion is borne out by the following stalagmometer readings obtained with a crude solution of insulin after digestion with varying amounts of charcoal:—

Grams charcoal.	0.0	0.2	0.4	0.6
Drops of solution	83	79	75	72

⁷ Moloney and Findlay: Trans. Roy. Soc. Canada, 17, Sec. V, 77 (1923).

Our first successful results were obtained by the method of Group IV, viz; by leaching a complex containing four units of insulin with 0.3 N solutions of fatty acids in aqueous alcohol; the fatty acid was removed by extraction with ether and the aqueous solution injected into rabbits.¹ The table gives the blood sugar readings observed; from these it may be deduced that by means of lauric acid three quarters of the sorbed insulin has been recovered, about one quarter by caprylic and heptylic acids and very little by the others. A parallel series with the sodium salts showed a slight recovery with the laurate and none with the others.

<i>Blood Sugar</i>		
	<i>Normal</i>	<i>2 hrs. after injection</i>
Butyric acid	0.118	0.104
Caproic	0.118	0.104
Heptylic	0.110	0.072
Caprylic	0.124	0.072
Lauric	0.118	0.054 (convulsions).

²There can be little doubt that the removal of the insulin is due to its replacement by the fatty acid. In the first place Bartell and Miller³ have shown that in soap solutions it is the acid liberated by hydrolysis which is sorbed by charcoal. This accounts for the greater efficiency of the acids compared with their salts in the recovery of insulin. Then the same authors have shown that the higher fatty acids are more readily sorbed by charcoal than the lower and our results show that their ability to liberate insulin increases in the same direction.⁴ Finally our experiments with alcoholic solutions of oleic acid, stearic acid, sodium oleate and castille soap gave results as good or better than those with lauric acid, while by the use of benzoic acid or of salicylic acid (which according to Bartell and Miller are very strongly sorbed by charcoal) practically all the insulin can be recovered. Glycocol, (not sorbed by charcoal) tartaric acid (sorption about the same as butyric), hydrochloric, nitric or sulphuric acids (sorption less than acetic), gave no yield, as was to be expected; the same is true of citric acid, alanine, potassium iodide and potassium nitrate. Phenol gave no yield, but an alcoholic solution of thymol recovered 75%, and so did an aqueous solution of ammonia, which is strongly sorbed by charcoal and a solution of bile salts whose power to greatly decrease the surface tension of aqueous solutions has long been known.

¹ Caution must be observed in interpreting the results of such experiments, to avoid the use of reagents which themselves lower blood sugar, such for example as sodium dihydrogen phosphate, potassium iodide, guanidine sulphate (Collip: J. Biol. Chem. 1923).

² We found that the dyes methylene blue, safranin and methy violet, which are readily sorbed by charcoal may be recovered by the use of soap solutions. In the case of the last two named the acidity of the solution from which the dye was sorbed does not affect its subsequent recovery; but if methylene blue be sorbed from solutions of pH 2.2 (phthalate buffer) it cannot be removed by aqueous soap solutions, while if sorbed at pH 3.8 part at least can be recovered.

³ J. Am. Chem. Soc. 45, 1106 (1923)

⁴ Warburg's work shows that in other instances the higher members of a series are the better replacing agents. (Z. Elektrochem, 28, 70 (1922)).

The following process based on the experiments referred to above has proved successful in the manufacture of commercial Insulin in these Laboratories¹:—Ten litres of a partially purified solution of insulin are set at pH 2.5 with hydrochloric acid and left overnight with 400 grams of charcoal with occasional stirring.² The charcoal is then removed, washed with water, and stirred with two litres of a 5% solution of acetic acid in 60% alcohol—this reagent leaches off certain impurities but leaves the insulin. After filtration the charcoal is digested for several hours at room temperature with 3.5 litres of a 12% solution of benzoic acid in 60% alcohol, and the insulin recovered from the solution by evaporating off the alcohol and removing the benzoic acid by ether—after which treatment of course the ether remaining dissolved in the insulin solution must be got rid of by evaporation. We are at present employed in studying the effect of higher temperatures during sorption and recovery; it appears that thereby the time required for the various processes can be materially reduced.

As it seemed likely that reagents which replace insulin from its complex with charcoal might also replace it from its complex in the pancreas, we tried extraction of the glands with alcoholic solutions of benzoic acid and obtained yields up to 2,000 units per pound of gland.³ Parallel runs with N/20 sulphuric acid (the reagent used by Doisy, Somagyi and Schaffer⁴) gave only 100-300 units per pound; and in the light of our experiments with charcoal cited above it seems probable that most of this must be ascribed to the action of the fatty acids liberated by the sulphuric from the gland.

The paper presents a classification of the methods available for recovering adsorbed substances from charcoal, etc., with a view to their isolation and purification; gives instances gathered from the literature of their application for such purposes and details their use in the purification of insulin.

Our thanks are due Professor W. Lash Miller for assistance in putting this paper in form.

*University of Toronto,
Connaught Laboratories,
December, 1923.*

¹ Best and Scott: *J. Biol. Chem.*, **57**, 709 (1923).

² If charcoal be used on an insulin solution which is deteriorating, the rate of destruction is increased.

³ The crude product so obtained is very unstable; methods of purifying it further are being studied.

⁴ *Proc. Am. Soc. Biol. Chem. Toronto meeting*, December, (1922).