(Abbildung des Diagramms in WUNDERLY und WUHRMANN[1]); ferner die UV-Absorption identisch gefunden mit jener von kristallisiertem Rinderalbumin (Armour & Co., Chicago). Nach E. COHN und Mitarbeiter[2] enthält Reinalbumin höchstens 0,04% Cholesterin, ein Gehalt, der zu gering ist, um meßbare lipophile Wirkungen hervorzubringen.

Die Hautfasermembranen werden für 48 Stunden bei Zimmertemperatur im wasserklaren Albuminsol belassen; darauf wird der Farbstoffgehalt der Elutionsflüssigkeit in 1-cm³-Küvetten des Stufenphotometers von Zeiß bestimmt. Als Vergleichslösung dient eine 0,166%-Albuminlösung. Für die blauen Farbstoffe wird Filter S_{61} verwendet, für Sudanrot S_{50}.

Tabelle I

Farbstoff	γ Farbstoff an der Membran	Elution nach 48 Std.	
		in % des gesamten Farbstoffes	γ Farbstoff pro mg Albumin
Fettfarbstoffe:			
Blau B.Z.L.	920	1,10	0,20
Sudanschwarz B	360	0,58	0,04
Sudanrot I	700	3,41	0,47
Wasserlösliche Farbstoffe:			
Evans-Blue, sauer . . .	285	57	3,2
Benzoblau, sauer	272	27	1,5
Trypanblau, sauer . . .	285	15	0,8
Victoriablau, basisch . .	136	8	0,2

Die Zahlen der letzten Kolonne geben eine Vergleichsmöglichkeit der lipophilen und der hydrophilen Eigenschaften von Reinalbumin. Wir sind gegenwärtig damit beschäftigt, diese Charakterisierung mit Patientensera durchzuführen, wo die lipoidtragenden Unterfraktionen der α- und β-Globuline pathologisch vermehrt sind.

Untenstehend ist das Ergebnis der Prüfung der eluierenden Wirksamkeit einer Konzentrationsreihe von Albumin. Vergleichshalber wurden gehaltgleiche Lösungen von «Subtosan» mitgemessen. «Subtosan» ist ein französisches Plasmaersatzmittel, dem ähnlich wie bei «Periston» das hydrophile, kolloide Polyvinylpyrrolidon (zyklisches Säureamid) als Basis dient. Der mittlere Farbstoffgehalt der Membran war 660 γ Sudanrot I.

Tabelle II

Elutionsmittel in 30cm³ NaCl phys. gelöst		Elution bei 18° C	
		nach 48 Stdn.	nach 120 Stdn.
		γ Sudanrot pro mg Elutionsmittel	
200 mg	Albumin	0,24	0,37
	Subtosan		0,09
400 mg	Albumin	0,17	0,25
	Subtosan		0,08
600 mg	Albumin	0,13	0,19
	Subtosan		0,08
800 mg	Albumin	0,11	0,16
	Subtosan		0,07

[1] CH. WUNDERLY und F. WUHRMANN, Brit. J. Exp. Path. *28*, 286 (1947).

[2] E. COHN *et al.*, J. Amer. Chem. Soc. *68*, 459 (1946).

Mithin ist die eluierende Wirkung der verdünnten Lösungen von Albumin größer als die der gehaltreichen. Demgegenüber bleiben die lipophilen Eigenschaften von «Subtosan» stark zurück, was für seine klinische Verwendungsmöglichkeit nicht ohne Bedeutung ist.

CH. WUNDERLY

Medizinische Universitätsklinik Zürich, den 10. Februar 1950.

Summary

In order to gain a model for the standard elution of the fat-dyes: Sudanred, Sudanblack, and Blue BZL, the latter are adsorbed on membranes of fibrous tissue. The following elution of the adsorbed fat-dyes by Albumin (human), as well as the french plasma Substitute "Subtosan", are followed colorimetrically. The results are compared with the elution of the hydrophile dyes: Evans-Blue, Benzoblue, Trypanblue, and Victoriablue. Such a comparison shows, that the elution of hydrophilic dyes by albumin is appreciably stronger than the elution of lipophilic dyes. The elution of Sudanred by Albumin is between 2 and 4 times stronger than by Subtosan.

Study of Some Constituents of Vitamin B_{12}

Through the kindness of Prof. T. REICHSTEIN and Dr. O. SCHINDLER of the University of Basle, who placed several specimens of crystalline vitamin B_{12} (prepared by them from liver) at our disposal, we were able to carry out a number of orienting experiments on the composition of this compound. Filter paper chromatography was employed in all experiments in the course of which a total of about 10 mg of the vitamin was used.

In view of the rather obscure relationship between vitamin B_{12} and the desoxyribose nucleosides[1] it appeared of interest to search for the presence of pyrimidines and of other nucleic acid constituents, such as desoxypentose, in hydrolysates of the vitamin. Several hydrolysis experiments were carried out in which the compound was degraded with concentrated formic acid (2 hours at 175° in a bomb tube), i. e. under conditions that bring about the complete cleavage of nucleic acids to free purines and pyrimidines[2]. The hydrolysates were examined chromatographically with some of the solvent systems (*n*-butanol-water, *n*-butanol-diethylene glycol-water with and without NH_3) that are normally used for the separation of purines and pyrimidines[3]. Test mixtures of the purines and pyrimidines were run alongside. Neither thymine nor any other pyrimidine or purine could be detected nor, for that matter, any compound giving the characteristic absorption shadow on the paper chromatogram in the ultraviolet light of the "Mineralight" lamp (Ultraviolet Products Corp., Los Angeles, Cali-

[1] W. SHIVE, J. M. RAVEL, and R. E. EAKIN, J. Amer. Chem. Soc. *70*, 2614 (1948). – L. D. WRIGHT, H. R. SKEGGS, and J. W. HUFF, J. Biol. Chem. *175*, 475 (1948). – W. SHIVE, J. M. RAVEL, and W. M. HARDING, J. Biol. Chem. *176*, 991 (1948). – C. E. HOFFMANN, E. L. R. STOKSTAD, A. L. FRANKLIN, and T. H. JUKES, J. Biol. Chem. *176*, 1465 (1948). – V. KOCHER and O. SCHINDLER, Intern. Z. Vitaminforsch. *20*, 441 (1949). – E. KITAY, W. S. MCNUTT, and E. E. SNELL, J. Biol. Chem. *177*, 993 (1949). – R. M. TOMARELLI, R. F. NORRIS, and P. GYÖRGY, J. Biol. Chem. *179*, 485 (1949).

[2] E. VISCHER and E. CHARGAFF, J. Biol. Chem. *176*, 715 (1948). – E. CHARGAFF, E. VISCHER, R. DONIGER, C. GREEN, and F. MISANI, J. Biol. Chem. *177*, 405 (1949).

[3] E. VISCHER and E. CHARGAFF, J. Biol. Chem. *176*, 703 (1948).

fornia)[1]. It should be mentioned that the inspection of chromatograms of such formic acid hydrolysates (irrigated with *n*-butanol-water) in ultraviolet light revealed the presence of two (and sometimes three) fluorescing zones of which the slower exhibited a yellowish, the faster a light-bluish fluorescence. Eluates with 0·1 N HCl showed, however, no characteristic absorption in the ultraviolet, but only a continuous increase in extinction towards the shorter wavelengths. These zones probably represent degradation products of the substituted benzimidazoles previously isolated from vitamin B_{12}[2].

The search for the presence of a desoxypentose made use of the procedure originally elaborated for the release of desoxyribose from desoxyribonucleic acids, which is based on the enzymatic digestion of the substance to the nucleoside stage by means of an *Aspergillus* enzyme preparation "mylase P" (Wallerstein Laboratories, New York), followed by a brief hydrolysis at p_H 1·5[3]. There were no indications that during a hydrolytic cleavage of this kind the liberation of a sugar took place.

In another type of chromatographic experiment the action of resting cells of *E. coli* on solutions of the vitamin (4 hours at 36° in 0·1 M phosphate buffer of p_H 6·9) was examined[4]. Here, too, no release of purines, pyrimidines or their derivatives was observed.

Several hydrolysates of the vitamin at 100° (N HCl for 30 minutes and one hour, 2 N HCl for two hours, N HPO_3 for 30 minutes) were examined for the presence of reducing sugars capable of yielding adsorption zones fluorescing in ultraviolet light following condensation with *m*-phenylenediamine[5]. The hydrolysates were chromatographed in aqueous butanol-pyridine, butanol-diethylene glycol, and isobutyric acid[6]. Although faintly fluorescent zones were observed in several instances, no clear evidence of the presence of a reducing sugar was obtained.

Finally, the amino compound giving the ninhydrin reaction[7] was investigated with the use of the quantitative micro-procedures recently developed in this laboratory in connection with studies of lipid composition[8]. Whereas tests for the occurrence of a compound of the type of choline, yielding an insoluble phosphomolybdate, were negative, no difficulty was experienced, in contrast to a statement in the literature[9], in demonstrating the presence in the chromatograms of the vitamin hydrolysates of one fraction reacting with ninhydrin to give a purple spot. In descending unidimensional paper chromatograms this component had a R_F value of 0·45 (as compared with 0·35 for ethanolamine) in *n*-butanol-diethylene glycol-water (4:1:1). When the upper layer of a mixture of 4 volumes of *n*-butanol, 1 volume of glacial acetic acid and 1 volume of water was employed as the solvent system, the R_F value of this component was 0·44 (ethanolamine 0·33). In agreement with the findings of ELLIS *et al.*[1] it is felt that this compound could be 2-amino-1-propanol. As chromatographic and analytical reference substance *dl*-2-amino-1-propanol, prepared by the reduction of *dl*-alanine ethyl ester with lithium aluminum hydride[2], was employed; but no statement can, of course, as yet be made regarding the optical configuration of the natural product. The quantitative determinations were performed by an adaptation of the method originally developed for ethanolamine[3]. The vitamin B_{12} crystals lost 10·5% of their weight (2 determinations gave 10·53, 10·55%), when dried for 2 hours at 61° over P_2O_5 in a high vacuum. The analyses reported here are based on the weight of the dry compound.

The most consistent results were obtained when the vitamin was hydrolyzed with 6 N HCl for 6 hours in a sealed tube at 100°. In two independently prepared hydrolysates, each analyzed several times, 13·3 and 13·5% of 2-aminopropanol were found. On the basis of a content of 2·65% P in the dried crystals of the vitamin[4] this corresponds to a molar ratio of aminopropanol to phosphorus of 2.

With a milder hydrolysis at 100° (1 hour in N HCl or 2 hours in 2 N HCl) the release of amino alcohol appeared incomplete; the corresponding molar ratio was about 1·4. A more energetic treatment (6 N HCl for 2 hours at 175° in a bomb tube) gave inconsistent results.

It should be pointed out that no definite claim as to the identification of the amino compound responsible for the ninhydrin reaction can be made on the basis of the experiments reported here. To give one out of possibly several examples: One other aminopropanol tested by us, namely 1-amino-2-propanol, occupies a position on the chromatograms that is indistinguishable from that of its isomer. The color values of the reaction products of these two amino alcohols with ninhydrin are practically identical. The molar ratio with respect to phosphorus would, therefore, remain the same, regardless of which of these isomeric aminopropanols occurs in the vitamin.

The acid hydrolysis of vitamin B_{12} has been reported to give rise to the formation of ammonia[1]. It was known from previous experience with other compounds that under the conditions of the chromatographic experiments the bulk, if not all, of the ammonia assembled in the ethanolamine region (R_F about 0·30 in butanol-diethylene glycol-water), forming a diffuse colored zone on the ninhydrin-treated papers. It was possible to exclude the possibility that this diffuse adsorption zone contained ethanolamine. Eluates of segments of this zone were, consequently, assayed photometrically after reaction with ninhydrin[5] and the results read against ammonia standards. With 6 N HCl (6 hours at 100° in a

[1] E. CHARGAFF, B. MAGASANIK, R. DONIGER, and E. VISCHER, J. Amer. Chem. Soc. *71*, 1513 (1949).

[2] N. G. BRINK and K. FOLKERS, J. Amer. Chem. Soc. *71*, 2951 (1949). – G. R. BEAVEN, E. R. HOLIDAY, E. A. JOHNSON, B. ELLIS, P. MAMALIS, V. PETROW, and B. STURGEON, J. Pharm. Pharmacol, *1*, 957 (1949).

[3] E. CHARGAFF, E. VISCHER, R. DONIGER, C. GREEN, and F. MISANI, J. Biol. Chem. *177*, 405 (1949). – E. VISCHER, S. ZAMENHOF, and E. CHARGAFF, J. Biol. Chem. *177*, 429 (1949).

[4] E. CHARGAFF and J. KREAM, J. Biol. Chem. *175*, 993 (1948).

[5] E. CHARGAFF, C. LEVINE, and C. GREEN, J. Biol. Chem. *175*, 67 (1948).

[6] S. M. PARTRIDGE, Biochem. J. *42*, 238 (1948).

[7] B. ELLIS, V. PETROW, and G. F. SNOOK, J. Pharm. Pharmacol. *1*, 60, 950 (1949).

[8] E. CHARGAFF, C. LEVINE, and C. GREEN, J. Biol. Chem. *175*, 67 (1948). – C. LEVINE and E. CHARGAFF, Federation Proc. *8*, 219 (1949); and unpublished results.

[9] N. G. BRINK, D. E. WOLF, E. KACZKA, E. L. RICKES, F. R. KONIUSZY, T. R. WOOD, and K. FOLKERS, J. Amer. Chem. Soc. *71*, 1854 (1949).

[1] B. ELLIS, V. PETROW, and G. F. SNOOK, J. Pharm. Pharmacol. *1*, 950 (1949).

[2] P. KARRER, P. PORTMANN, and M. SUTER, Helv. chim. acta *31*, 1617 (1948).

[3] C. LEVINE and E. CHARGAFF, Federation Proc. *8*, 219 (1949); and unpublished results.

[4] Private communication from Prof. T. REICHSTEIN.

[5] S. MOORE and W. H. STEIN, J. Biol. Chem. *176*, 367 (1948).

sealed tube) 6% of ammonia were found; with milder hydrolysis methods the amounts of NH_3 varied from 2·6 to 3·8%.

This work has been supported by a grant from the *Life Insurance Medical Research Fund.*

ERWIN CHARGAFF, CELIA LEVINE, CHARLOTTE GREEN, and JACOB KREAM

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York 32, N. Y., February 28, 1950.

Zusammenfassung

Es wird über eine Reihe von Studien berichtet, welche die Zusammensetzung des Vitamins B_{12} zum Gegenstand haben. Diese mit sehr kleinen Substanzmengen ausgeführten Versuche bedienten sich ausschließlich der Chromatographie auf Filtrierpapier. Zwei Probleme bildeten den Hauptgegenstand der Untersuchung, nämlich die Frage des Vorkommens von Nukleinsäurebestandteilen im Vitamin und die quantitative Bestimmung der schon früher nachgewiesenen Aminoverbindung. In der für Nukleinsäuren ausgearbeiteten Versuchsanordnung konnten weder Purine, noch Pyrimidine oder Desoxyribose nachgewiesen werden. Die Bestimmung der Aminoverbindung ergab das Vorliegen von 2 Molen Aminoderivat (berechnet als 2-Amino-1-propanol) pro Mol Phosphor.

Studies on the Possibility of Using Protamin Sulfate in the Technique of *in vitro* Cultures of Hematopoietic Tissues

The use of heparin (CRACIUN[1]) has rendered easier the technique of the culture of tissues, because it allows of obtaining a fluid plasma—with unaltered physical and chemical conditions—to be used as a cultural medium. At the appropriate moment this heparinized fluid plasma is then coagulated by the addition of embryonic extract.

In order to simplify this second phase of the cultural technique, which depends on embryonic extracts of recent preparation (these extracts lose their activity in 1–2 days), we have thought useful to investigate whether the part played by these extracts could be taken over by some stuff easy to store, and consequently always at the disposal of the scientist. Such a stuff would be desirable especially for cultures of leucocytes and hematopoietic tissues in general, which themselves secrete trephonic substances into the medium.

As a means for coagulating heparinized plasma we have chosen to study the proprieties of protamin sulfate, whose ability to neutralize heparin had been shown by CHARGAFF and OLSON[2]. On the one hand we investigated not entirely clear some aspects of the interference of protamin sulfate with heparinized plasma, and on the other hand the behaviour of the various functional activities of bone marrow cultures in heparinized plasma coagulated with protamin sulfate instead of embryonic extracts. In this paper we summarize some of the results that concern the first of the two aspects of this question.

First we set out to find the amount of protamin sulfate (Protamin 3711/2 Roche) necessary to coagulate *in vitro* human plasma of healthy subjects fasting since 12 hours, this plasma having been obtained and kept fluid by the addition of 10 AU (anticoagulant units) of heparin (Liquemin Roche) per cc. of blood. At the same time we determined the time necessary for clotting when the amounts of protamin varied in relation to the optimal amount. We have chosen 10 AU of heparin per cc. of blood, because this is the dose which we generally use for the preparation of the cultural medium in our studies of cultures of hematopoietic tissues. We have been able to establish the following points:—

(1) With amounts of protamin less than 0·001 mg no clotting takes place—at least within the 48 hours we prolonged our observation.

(2) At 37° C coagulation begins with a dose of 0·001–0·005 mg of protamin, and can be obtained with all the intermediate amounts up to the extreme one of 3 mg.

(3) With an amount of protamin greater than 3 mg, the plasma does not coagulate any more, at least within 48 hours.

(4) The optimal amounts of protamin by which the fluid plasma is coagulated within 15′ extend from 0·03–0·6 mg; those by which the clotting is obtained within 30′ range between 0·02 mg and 1 mg.

(5) When the amounts of protamin depart from the optimal ones—increasingly or decreasingly—the clotting time is obviously increased. For instance, our minimal coagulating amount of protamin has been 0·001 mg with a coagulation time of 115′: the maximal coagulating amount of protamin has been 3 mg, and coagulation time was 840′.

The second point we wanted to investigate was the influence of temperature on the clotting time *in vitro* of human heparinized plasma under the effect of protamin. We have been able to note that:

(1) The optimal temperature for clotting to happen lies between 33°–37° C.

(2) At room temperature the clotting time is twice or three times as great as at optimal temperature.

(3) The influence of temperature on coagulation becomes more marked when amounts under and over the optimal protamin quantities are used.

As a third aspect of the problem we wanted to investigate whether protamin is active only on recently prepared plasma, or whether it is able to coagulate also fluid plasma stored at room temperature. We established that upon addition of optimal doses of protamin the clotting time increases (up to 2–3 times the initial one) up to complete absence of clotting on the 3rd–5th day. For the plasma stored in ice-box we have been able to note the persistence of the coagulability till 9th day.

Experiments made with heparinized plasma of guinea pig and rabbit fasting since 18 hours showed that the optimal protamin amounts and coagulation times for the plasma of these animals correspond to the values established for human plasma.

At last we tried to assembly some data in order to explain the fact that protamin is able to clot plasma obtained and preserved fluid by heparin addition, although it is itself an anticoagulant (THOMPSON[1], WALDSCHMIDT-LEITZ and coworkers, 1929). We have therefore repeated the titration between heparin and protamin (CHARGAFF[2]), by titrating 5 cc. of a heparin solution containing 1,000 AU/cc. with a 1:1,000 protamin

[1] E. CRACIUN, Arch. exp. Zellforschg. *2*, 295 (1925/26).
[2] E. CHARGAFF and K. B. OLSON, J. Biol. Chem. *122*, 153 (1937/38).

[1] W. H. THOMPSON, Hoppe-Seylers Z. physiol. Chem. *29*, 1 (1900).
[2] E. CHARGAFF, J. Biol. Chem. *25*, 671 (1938).