## 3 遗传信息的载体：DNA

基础研究通过推进人类认知前沿可能解决人类面临的问题；人类面临的问题通过刺激科学研究可能推进人类认识前沿。遗传的科学问题与肺炎的医疗问题合力揭示了基因的物质基础。

科学前沿可以出现不同研究途径的意外交汇。从生物功能的角度诞生了遗传学：1866 年 Mendel 开创遗传学，1880 年德国的 Flemming 发现染色体，1902 年德国的 Boveri 获证据支持染色体为遗传的物质基础，1910 年后美国的 Morgan 及其学生丰富和发展了染色体的遗传学说。从化学成分的角度出现了生物化学：1869 年，瑞士的 Miescher 在研究伤口脓细胞化学成分的过程中发现核素，其后德国的 Kossel 发现了核酸中的嘌呤和嘧啶，二十世纪初美国的 Levene 确定以核苷酸连接为基础的核酸一级结构，瑞典的 Caspersson 等证明染色体含核酸和蛋白质，但当时研究 DNA 生物化学和生物物理的专家以为 DNA 无特异性、缺乏信息携带能力。

1928 年，研究肺炎致病性细菌的过程中，英国的 Griffith 分析不同类型病例分布后推测不同型的肺炎球菌可能会变化，之后设计实验发现了转化现象。1944 年，美国洛克菲勒医学研究所的 Avery、MacLeod 和 McCarty 研究转化的物质基础，提出脱氧核糖核酸（DNA）是改变细菌可遗传特性的转化因子。其后验证 DNA 的转化活性、证明 DNA 有特异性、发现转化活性不局限于特定细菌的特定性状。

DNA 是遗传的物质基础这一概念，刺激了进一步研究。其中最重要的工作是，在 Franklin 和 Wilkins 对 DNA 进行X 线衍射实验的基础上， Watson 和 Crick 于 1953 年提出了 DNA 双螺旋结构模型，分子生物学由此而诞生。

## 3.1 核酸及其化学结构

### 3-1-1 核酸的发现

米歇尔（Johann Friedrich Miescher，1844-1895）出生于科学世家，父亲曾任瑞士的 Basel 大学生理学教授、舅舅 Wilhelm His（1831-1904）为著名解剖学家。1868 年春，米歇尔毕业于巴塞尔医学院后，因不感兴趣行医、自己听觉有问题、而舅舅认为“组织发育的剩余问题只能依据化学基础来解决”（Dahm，2005，2008），米歇尔到德国图宾根接受科学训练。他在有机化学实验室工作一学期后转入 Felix Hoppe-Seyler（1825-1895）实验室。Hoppe-Seyler 乃时称“生理化学”（后称生物化学）的先驱，他发现血红蛋白的可逆性氧化、并命名（hemoglobin），他命名蛋白质为 proteid（后称 protein）。Hoppe-Seyler 建议米歇尔研究淋巴细胞的化学成分，米歇尔因难以从淋巴结获足够量的纯化淋巴细胞，转而研究可大量获得的白细胞，其来源为外科诊所绷带上的脓。

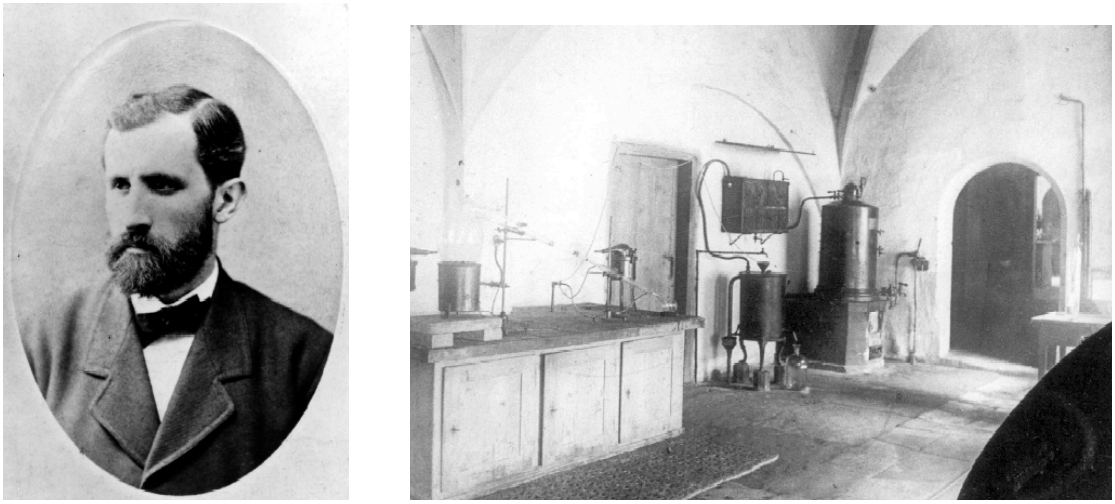


图 1. 左：Johan Friedrich Miescher（1844-1895）；右：他的实验场所，原为图宾根堡之厨房，照片是他做实验十年后拍摄

米歇尔起初关注白细胞的蛋白质，他意识到蛋白质和脂肪主要位于细胞质。

在研究过程中发现一种物质被酸沉淀、加碱中和后再溶于水，其特性不同于蛋白质和脂肪，他认为是新的物质，并猜测来源于细胞核。他探索了其他两种纯化细胞核的方法，包括用稀盐酸低温处理或用蛋白酶预处理。获得足量物质后，他分析其元素含量，发现氮占 14%、磷 5.8%、硫 1.8%。与当时其他物质相比，其磷含量特别高。低量的硫，我们后来知道，是蛋白质污染造成。米歇尔用氮和磷含量建立其发现的物质为不同于蛋白质和脂肪的新物质，他命名为核素（nuclein）。

1869 年，米歇尔离开图宾根 Hoppe-Seyler 的实验室到莱比锡，在那里写好论文于当年投稿给 Hoppe-Seyler 主编的杂志，后者对米歇尔的结果有怀疑，直到自己、两位学生（Pál Plósz 和 Nicolai Lubavin）重复米歇尔的实验后，Hoppe-Seyler 才于 1871 年在自己主编的医学化学杂志同时发表五篇核素的文章，首先是米歇尔的“脓细胞的化学组成”（Miescher，1871a；Dahm，2008）；其次是 Plósz 验证核素只存在于（鸡和蛇的）有核红细胞、而不存在于（牛的）无核红细胞（Plósz，1871）；第三篇为 Lubavin 在奶酪中发现核素（Lubavin，1871）；第四篇为 Hoppe-Seyler 完全肯定米歇尔的工作，并验证核素的磷含量高（Hoppe-Seyler，1871）；第五篇为米歇尔后投稿的一篇报道他在蛋黄中发现核素（Miescher，1871b）。

1871 年 Miescher 回 Basel，1872 年接父亲和舅舅任过的教职。在 Basel，他从莱茵河三文鱼的精子提取了大量核素（Miescher，1874）。他知道核素不仅在鱼，也在蛙、牛、鸡的精子中。1872 年至 1877 年，他提出核素中的磷都以磷酸形式存在，核素至少含有四种碱基（Levene and Bass, 1931）。

德国科学家 Richard Altmann （1852-1900）改进了核酸制备方法，产物无蛋白质，他还于 1889 年提出核酸的名词（Altmann，1889），米歇尔认为核酸和核

素相同，无需改名。

以化学分析为开端的核酸研究，起初不是为了特定生物功能的分子基础。Hoppe-Seyler 认为发现细胞核的物质很重要，米歇尔认为自己发现的新物质其重要性不亚于蛋白质。米歇尔发现精子中有核素后，提出“如果单个物质可以是受精的特异原因的话，那么无疑首先应该考虑的是核素”。但他又觉得不太可能是一种物质，其原因之一是核素好像不可能有很大的多样性，难以解释个体性状的多样性（Dahm，2005）。

### 3-1-2 核酸的化学分析

Hoppe-Seyler 在斯特拉斯堡大学建立了德国首个生物化学系。1872 年，Albrecht Kossel （1853-1927）听过 Hoppe-Seyler 的生理化学和病理化学课，1877 年 Kossel 毕业于 Rostock 大学并考完行医执照后，加入 Hoppe-Seyler 的实验室，1879 年开始发表有关核素的研究（Kossel，1879；Jones，1953），1883 年到柏林大学工作。1874 年，巴塞尔大学的 Jules Piccard 从精子的核素发现鸟嘌呤（guanine，G）和次黄嘌呤（hypoxanthine）。1880 年，Kossel 从酵母的核素中发现黄嘌呤（xanthine）。1885 年，Kossel 从酵母核素中发现腺嘌呤（adenine，A）。Kossel 用 Altmann 的制备方法进一步分析，1891 年宣布发现核酸含磷酸、腺嘌呤、鸟嘌呤（Kossel，1891）。1893 年他和 Neumann 发现核酸含胸腺嘧啶（thymine，T），1894 年他们发现核酸含胞嘧啶（cytosine，C）。1900 年 Kossel 的学生 Ascoli 发现尿嘧啶（uracil）（Levene and Bass， 1931；Jones，1953）。Kossel 还研究了蛋白质，1884 年发现细胞核中的组蛋白（histone，是二十一世纪才热门的蛋白质），1896 年 Kossel 发现一个常见的氨基酸：组氨酸（histidine）。

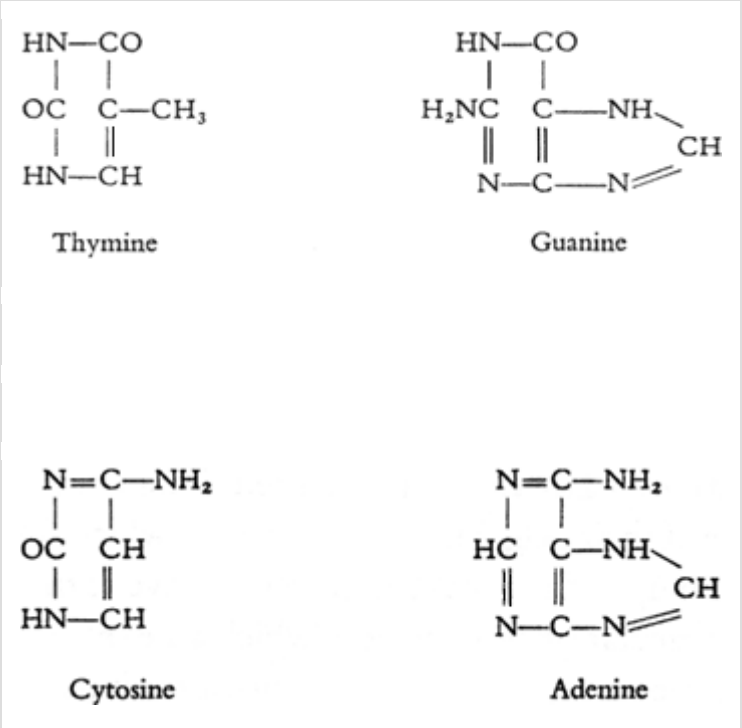



图 2. 胸腺嘧啶、胞嘧啶、鸟嘌呤、腺嘌呤的化学结构，自 Kossel 诺贝尔演讲

3-1-3 核酸的化学结构

二十世纪上半叶的核酸生物化学专家 Phoebus Levene（1869-1940）出生于核素被发现的 1869 年。他在俄国圣彼得堡念过军事医学院，因俄国排犹而随家人移民美国、在纽约行医，因感兴趣研究而在哥伦比亚大学注册念书，也设法获得研究训练，1896 年在纽约州医院病理研究所生理化学实验室初次接触核酸。他多次到欧洲进修，曾到德国分别跟随 Kossel 和 1902 年诺贝尔化学奖得主 Emil Fisher（1852-1919）。1901 年 John D Rockefeller（1839-1937）斥资在纽约建立与法国巴斯德研究所相媲美的洛克菲勒医学研究所。1905 年 Levene 被第一任所长 Simon Flexner 聘为助理，1907 年成正式研究员、并负责化学部直至 1940 年去世。Levene 一生发表过七百多篇论文，研究过核酸、蛋白质、氨基酸、脂、碳水化合物等。

1901 年，Levene 发现不同来源的核酸不是都含 4 种嘌呤、而只含 A 和 G 两种。1906 年，Steudel 也同意胸腺核酸只含两种嘌呤，且等分子数（equimolecular）。C 和 T 两种嘧啶不是嘌呤的衍生物而是核酸所含的原始碱基，也是二十世纪初经过争论和实验所验证（Levene and Bass，1931）。1903 年，Levene 发现酵母核酸含 U 不含 T。1909 年，Levene 认为酵母核酸含两种嘌呤、两种嘧啶。

Levene 发现了核酸中的核糖（ribose）、脱氧核糖（deoxyribose），碱基与核糖连接为核苷（nucleoside），再接磷酸为核苷酸（nucleotide）。

Levene 提出了核酸的化学结构（现称一级结构）：RNA（当初谓“酵母核酸”yeast nucleic acid）由 A、G、C、U 四种核苷酸链接组成（Levene，1909，1917a），DNA（当初谓“胸腺核酸”thymus nucleic acid）由 A、G、C、T 四种核苷酸（nucleotides）共价键相连而成（Levene and Jacobs，1912，1929）。1935 年，Levene 等提出了 DNA 和 RNA 正确的化学链接（Levene and Tipson, 1935）。

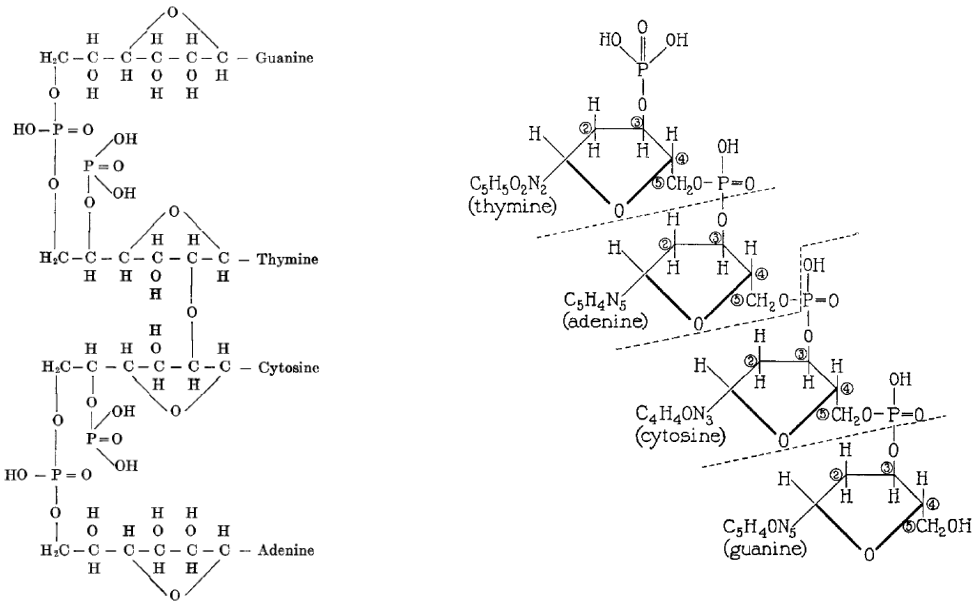



图 3. 左：Levene 和 Jacobs 于 1912 年提出的 DNA 的化学结构；右：Levene 和 Tipson 1935 年提出的正确的 DNA 中化学链接

Levene 最早于 1909 年提出 RNA 含四种核苷酸，提到它们为等分子数。四核

苷酸假说较强调核酸由四种核苷酸组成是反驳德国的 Hermann Steudel 和美国霍普金斯大学的 Walter Jones 等提出核酸只含三核苷酸、二核苷酸（Levene，1919，1920a，1920b）。Levene 还认为黄嘌呤和次黄嘌呤是实验过程的次生产物，非核酸原始成分，这样 DNA 只有 A/G/C/T、RNA 只有 A/G/C/U。1912 年 Levene 提出 DNA 的结构时显示了四种核苷酸，但未提四种核苷酸的相对含量（Levene and Jacobs，1912）。Mandel 和 Levene (1905)用检测脾的核酸发现 A/G/C/T 的含量不同、乳腺的核酸中四种碱基也不同。但 Osborne 和 Harris（1902）检测认为麦芽核酸中 A 和 G 等分子数，后来其他人和 Levene（Levene and Mandel, 1908; Levene, 1909）也认为核酸含碱基为等分子数，Levene 在 1917 年用“四核苷酸理论”(Levene, 1917a)、1931 年叙述“四核苷酸结构”认为 DNA 链中各种核苷酸的含量相同（Levene and Bass, 1931）。1930 年代以前还误认为核酸只是四个核苷酸组成的小分子，未意识到其为分子量很大的多聚体。到 1938 年知道核酸分子量几十万到百万道尔顿后，Levene 和其他人还认为核酸可以是四核苷酸不断重复的多聚体。

## 3-2 核酸与染色质

### 3-2-1 核酸的亚细胞定位

1914 年，德国的 Robert Feulgen（1884-1955）发现 DNA 在溶液中通过盐酸（暴露出 DNA 的醛基）和 Schiff 试剂（品红亚硫酸，可与醛基反应）两步可显紫红色， RNA 不能显色，后称 Feulgen 反应（Kasten，2003）。

1923 年，Feulgen 将这一反应引入组织化学：直接在生物的组织切片上进行反应，以此确定 DNA 在组织或细胞的存在部位。

1924 年他和技术员 Heinrich Rossenbeck 以此方法检测多种动植物组织、细胞后证明 DNA 存在于细胞核，不仅动物细胞核、而且植物细胞核（Feulgen and

Rossenbeck，1924）。

Feulgen 也改变了前人误以为 DNA（胸腺核酸）存在于动物、RNA（"酵母核酸"）存在于酵母和植物的误解，而从酵母中提取 DNA 还要到 1948 年（Chargaff and Zamenhof, 1948）。

瑞典卡罗琳斯卡医学院的 Torbjörn Caspersson（1910-1997）发现核酸对 260nm 紫外线有最佳吸收峰（Casperson，1932，1936），Morgan 最后的研究生 Jack Schultz（1904-1971）与 Caspersson 用紫外检测证实 DNA 定位于细胞核（Schultz and Caspersson，1940）。

### 3-2-2 核酸与染色质

Schultz and Caspersson 还观察到果蝇唾液腺多线染色体条带变化后核酸含量变化、果蝇卵母细胞染色体数量变化可以改变核酸含量（Caspersson and Schultz，1938）。

Caspersson 与同事 Einar Hammarsten（1889-1958）合作分析染色体的核酸和蛋白质组分，用蛋白酶消化蛋白质后得到高纯度的核酸（Caspersson，Hammarsten，Hammarsten，1935）。他们观察到果蝇多线型染色体条带与核酸的关系非常逼近核酸与遗传的关系。

Hammarsten 和 Caspersson 发现 DNA 不是短链、而是长链，分子量很大（50 万到 100 万），所含嘌呤环和嘧啶环的平面与链的长轴垂直（Signer, Caspersson, Hammarsten，1938）。英国 Leeds 大学纺织物理实验室的 William Astbury 通过 X 线衍射分析发现垂直于长轴的两个核苷酸之间距离为 3.34Å（Astbury and Bell，1938），Astbury 认为核苷酸间距与蛋白质中氨基酸间距很接近可能非巧合、而说明核酸链与蛋白链之间有关系。

1942 年，比利时的 Jean Brachet（1909-1988）用染色方法证明 DNA 在细胞核的染色体上，RNA 在动物细胞质与核仁中（Brachet，1942；Thomas，1992）。他用吡罗红（pyronin）和甲基绿（methyl green）混合染料，甲基绿染 DNA 显绿色，吡罗红染 RNA 显红色，两者都染显蓝色。当时俄国移民美国在洛克菲勒医学研究所工作的生物化学家 Moses Kunitz（1887-1978）利用 RNA 酶（RNase，降解 RNA）对热不敏感的特性，从牛胰腺提取到高纯度的 RNase（Kunitz，1940）。Brachet 用 RNase 处理组织切片，可以去除 RNA 的染色、只显 DNA 染色。他发现染色体含 DNA（可看到有条带的昆虫巨大染色体上 DNA 染色也呈条带，有些条带还含 RNA），细胞质含 RNA、且其含量与蛋白质含量有相关性。

至 1940 年代初期，确切知道 DNA 存在于细胞核的染色体上。不过，染色体上即能检测到 DNA、也能检测到染色体上的 RNA 和蛋白质，并不能仅仅由亚细胞定位确定 DNA 是遗传物质。

### 3-3 遗传物质是蛋白质还是核酸？

染色体既含核酸、也含蛋白质，携带遗传信息的分子究竟是什么？

二十世纪上半叶，已知蛋白质很重要。十九世纪提出酶为生物催化剂的概念，到二十世纪初争论酶是蛋白质还是其他分子。因研究叶绿体而获 1915 年诺贝尔化学奖的德国犹太科学家 Richard Willstätter（1872-1942）认为酶是蛋白质制备中的其他污染物质。其他科学家的工作，特别是 1926 年美国 Cornell 大学的 James Sumner（1887-1955）和 1930 年洛克菲勒医学研究所的 John Northrop（1891-1987）分别获得结晶纯的尿素酶和胃蛋白酶，证明酶的分子本质是蛋白质，获 1946 年诺贝尔化学奖。在这样的背景下，已知染色体有蛋白质和核酸时，很多人怕再次犯低估蛋白质重要性的错误（Judson，1979）。1934 年，英国的 J D Bernal

（1901-1971）和 Dorothy Hodgkin（1910-1994）第一次获得蛋白质（胃蛋白酶）的晶体结构（Bernal and Crowfoot, 1934），显示蛋白质的结构复杂性。此前已知蛋白质的生化特性和功能多种多样，人们易信蛋白质可以携带丰富的信息。

对细胞核的核酸与蛋白质都有研究的 Kossel 于 1910 年因为其蛋白质部分的工作而获奖，他于 1912 年发表的文章称要从蛋白质的化学特性理解发生细胞传递种系特异性（Kossel，1912）。核酸专家 Jones 称“生理化学家公认所有核酸与酵母和胸腺核酸之一相同”(Jones, 1914)。Levene 认为所有种属器官、组织来源的核酸结构不变，没有个性，没有特异性，不可能是孟德尔性状的携带者(1917b)。细胞生物学家 Edmund Wilson 在其权威教科书认为遗传物质是蛋白质不是核酸（Wilson, 1925)。四核苷酸假说到 1931 年比较强调四种核苷酸等分子数(Levene and Bass, 1931)，在 Signer, Caspersson, Hammarsten（1938）确定 DNA 分子量很大、是很长的链以后，仍未及时改变核酸像淀粉一样不太可能携带信息的错误观念。

用染色确定 DNA 存在于细胞核中的 Caspersson、Hammarsten、Brachet 皆未提出核酸是遗传的物质基础。Caspersson 认为染色体中的蛋白质可能是遗传物质（Caspersson，1936)。

1941 年 Schultz 认为按遗传学的预计基因应该是线性、有特异性、存在于染色体上、能自我复制、同时能影响细胞的合成代谢，而核酸与蛋白的复合体（nucleoprotein）符合这些条件、应该是基因的物质基础（Schultz，1941)。他在分别讨论染色体的核酸和蛋白质时，认为蛋白质确实有特异性，而核酸是否有特异性尚不清楚：虽然一般以为核酸单调，他指出当时分析过结构的核酸只有来源于胸腺的，不能排除不同细胞的核酸有特异性的可能性。1943 年，他指出病毒

肯定有基因，从病毒、细菌到高等动植物都有核酸和蛋白质复合体，而它们都能复制，所以细菌是否有细胞核并不重要，有核酸和蛋白质复合体为基础的基因。他依据已知三种含氨基酸不同的烟草镶嵌病毒（TMV）所含核酸在当时检测显示很恒定，提出应该是蛋白质给予病毒特异性、而核酸不能（Schultz，1943），所以最接近提出核酸是遗传基础的 Schultz 因当时检测手段的限制退而认为蛋白质是遗传物质。

### 3-4 肺炎球菌的分型和分类

十九世纪下半叶，法国的巴斯德（Louis Pasteur，1822-1895）和德国的科霍（Robert Koch，1843-1910）创立了现代微生物学：继承前人积累确立了细菌致病论（germ theory）、发现重要微生物、发明预防传染病的疫苗、培养微生物学家。

细菌是单细胞生物，但无典型的细胞核，所以称为原核生物（prokaryotes）。细菌细胞质外有细胞膜（plasma membrane），膜外有细胞壁（cell wall），有些细菌还有荚膜（capsule）。

细菌性肺炎长期困扰人类、在抗生素应用以前常导致死亡，二十世纪初美国每年因肺炎去世逾 5 万人。即使现在全世界每年也有上亿肺炎患者，肺炎是疾病导致儿童死亡的最大原因，每年近百万 5 岁以下儿童死于肺炎（Henriques-Normark and Tuomanen, 2013）。1918 年，著名内科医生 William Osler（1849-1919）称肺炎为“人类死亡的罪魁祸首”（captain of the men of death）。肺炎可以通过空气传染，其流行为国家所关注。

1881 年，美国细菌学家 George Sternberg（1838-1915）和法国细菌学家巴斯德分别发现肺炎球菌（*Streptococcus pneumoniae*）（Sternberg, 1881; Pasteur, 1881）。

1884 年，德国的犹太医生 Albert Fränkel（1864-1938）证明肺炎球菌为大叶性肺炎的致病菌。

### 3-4-1 肺炎球菌的分型

以抗原性（antigenicity）对肺炎球菌进行分型，原因是二十世纪初治疗肺炎是用抗血清（antiserum，它含抗体，antibodies），而治疗不同抗原性的肺炎球菌需要不同的抗血清，含不同抗原的肺炎球菌与不同的抗体反应。德国细菌学家 Friedrich Neufeld（1869-1945）提出这些抗体既可抑制特定类型的肺炎，又可用来将肺炎球菌分成 I、II、III 型（Neufeld and Händel, 1909，1912）。

美国洛克菲勒研究所附属医院的 Alphonse Dochez（1882-1964）发现肺炎球菌还有 IV 型，且 IV 型中还有多种（Dochez and Gillespie, 1913）。1913 年 Oswald Avery（1877-1955）加入洛克菲勒，1917 年他与 Dochez 等发现 IV 型更多种类（Avery *et al.*, 1917）。1922 年，英国卫生部病理实验室的 Fred Griffith（1879-1941）发现 IV 型中至少有 12 种不同的类型（Griffith，1922）。I 型和 II 型常致病强，IV 型致病弱、存在于有些正常人的口中，IV 型内不同株的差异较大、有些也有致病性。一般来说，一种肺炎球菌只含这四型的一种抗原。

不同抗原型的肺炎球菌因何不同？1917 年，Dochez 和 Avery 报道肺炎球菌的抗原不是肺炎球菌细胞破裂后从细胞内掉到细胞外的物质，而是细菌表面的物质，可溶于培养液和体液（Dochez and Avery，1917a），他们曾误认为是蛋白质（Dochez and Avery，1917b）。这些研究由 Dochez 开始，其后合作，一战以后 Dochez 入伍赴法国（Dubos，1976）。1918 年从美国 Kansas 首先爆发所谓“西班牙”流感带来的全球恐慌（最后估计感染 5 亿人，死亡数千万到一亿），Avery 与洛克菲勒医院都研究流感病毒。

1922 年，几经劝说后，化学家 Michael Heidelberger 加入 Avery 的肺炎研究（Heidelberger，1977）。他和 Avery 实验室其他人从 1923 年至 1934 年一系列实验阐明肺炎球菌分型的抗原不是蛋白质、而是多糖（polysaccharide）（Heidelberger and Avery, 1923; Avery and Goebel, 1933; Goebel, Avery and Babers, 1934），这些抗原为细菌细胞荚膜的完整性所需，也就对肺炎球菌的致病性重要，Avery 等的研究是免疫化学的重要工作。

Heidelberger 和 Avery 于 1923 年提出多糖、而非蛋白质决定细菌抗原特异性，在当时很不为大家接受，但到 1932 年后几乎每年因此获得诺贝尔奖提名（Reichard, 2002）。

4-2 肺炎球菌类的转换

细菌的类型能否变化？1880 年，巴斯德发现禽霍乱体外培养后致病性降低。1887 年，细胞免疫学创始人、俄国的 Ilya Metchnikoff（1845-1916）发现炭疽杆菌在抗血清中培养后致病性降低。1915 年南非的 AR Friel 发现肺炎球菌在抗血清培养后降低致病性并改变抗原性。1916 年，Avery 实验室的研究生 Laura Stryker 发现肺炎球菌的致病性经抗血清培养后下降，而进入动物体内可以恢复（Stryker, 1916）。1921 年，伦敦 Lister 研究所的 Joseph Arkwright（1864-1944）总结他研究的几种肠道细菌，同一种细菌不仅有致病性不同的类，而且形态不同：致病性强的表面形态光滑（smooth）为 S 类、致病性弱的表面粗糙（rough）为 R 类（Arkwright, 1921）。Arkwright 提出这些差别是遗传突变所致，S 和 R 类的存在多寡为达尔文的自然选择所定。

1922 年，Griffith 向英国卫生部报告 1920 年至 1922 年 150 例大叶性肺炎病人中肺炎球菌分型的情况（Griffith，1922）。1923 年，Griffith 向卫生部报告，

致病的（virulent）表面光滑（S 类），不致病的（avirulent）表面粗糙（R 类），它们有不同抗原性（可以分别有抗 S 的抗血清和抗 R 的抗血清）。在体外培养肺炎球菌时如有抗血清，肺炎球菌的致病性和形态会改变，从 S 类变成 R 类，还观察到 R 类变成 S 类（Griffith, 1923）。

1925 年，洛克菲勒的 Hobart Reimann 验证在血清或胆盐、甚至一般培养基中培养 I 型 S 类的肺炎球菌后，致病的 S 类细菌有些变成 R 类，失去致病性、改变形态和抗原性，但在他的实验中 R 类无论在体外还是动物体内都不能变成 S 类（Reimann，1925）。霍普金斯大学的 Harold Amoss 也验证了 Griffith 的结果：致病类的 I 型肺炎球菌，可以在体外培养变成不致病类；但 Amoss 未观察到不致病类回复为致病类（Amoss，1925）。因为 Reimann（1925）、Amoss（1925）用了分离的单菌落做实验，较 Stryker（1916）和 Griffith（1923）更有说服力，证明单个 S 类的菌变成 R 菌、而不是最初 S 类菌落中混有 R 菌个体。Reimann 还发现有些型的肺炎球菌也可以在动物（如兔、豚鼠、马，但非狗）体内从 S 类变成 R 类（Reimann，1927）。

### 3-5 肺炎球菌型间转化实验

1928 年，Griffith 在《卫生学杂志》上发表细菌转化的论文（Griffith, 1928）。首先，他分析了 1920 年至 1927 年的流行病资料，发现如果将病例分成三个时间段（1920 至 1922、1922 至 1924、1924 至 1927），那么随着时间推移，感染 I 型和 III 型的人数无规则性变化，但 II 型的减少（从 32.6%变为 7.4%）、IV 型的增多（30.0%变为 53.7%）。

他注意到 1922 年的病例中单个病人可以同时携带不止一型的肺炎球菌。这是因为病人多次感染，还是只有一次感染一型的肺炎球菌、其后细菌在病人体内

从一型变成另一型？1922 年到 1927 年病人群体中观察到的趋势支持变型的可能，但仅从相关性进行推测，缺乏直接证据。

Griffith 从 1922 年一位含多型肺炎球菌之病人的痰中获得 I 型肺炎球菌，接种老鼠可以致病，从老鼠中获得肺炎球菌再接种下一群老鼠，这样系列传代（serial passage），发现后来可以出现 IV 型的肺炎球菌，从而显示 I 型可以变成 IV 型。他再从多位病人获得 I 型肺炎球菌、在老鼠接种验证可以变成 IV 和 III 型，从多位病人获得 II 型肺炎球菌、在老鼠可以变成 III 型。

这时，他用典型的英国双重否定句：综合考虑可能性来看，型间转换假说的可能性似乎不比多重感染更不可能。

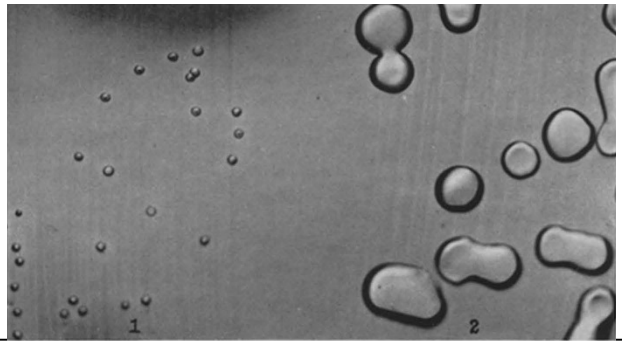



图 4. 左：不致病的 II 型 R 类肺炎球菌；中：致病的 III 型 S 类肺炎球菌；右：Fred Griffith （1879-1941）。左和中来自 Avery 等（1944 年）。

Griffith 认为已有证据还不足以区分型变与多重感染两种假说，需要继续实验。

他发现，一般来说无论肺炎球菌依据抗原分型是哪种（I、II、III 或 IV），都有致病的 S 类和不致病 R 类。无论哪种抗原分型，其致病的 S 类如果在老鼠传代接种，都可变成不致病的 R 类。也有两种方式可在体外培养让 S 类变成 R 类：一种是将某型的 S 类细菌（如 I 型的 S 类）在相应的抗血清中培养（如含 I 型抗血清），其后可获同型的非致病菌（如 I 型的 R 类）；另一方式是不加抗血清，在固体培养基中培养，传多代后也能从 S 类获 R 类。不过每次的菌株变化的情况

不同、传代后的菌株稳定性也不同，有些变成 R 类后可以很快回复为 S 类，有些变成 R 类后很稳定、难以回复为 S 类。回复变化只能在同一抗原分型之内，如：I 型的 R 类变成 I 型的 S 类，不出现 I 型的 R 类变成 II 型的 S 类。保存在冰箱但未被允许生长分裂的肺炎球菌不出现 R 类和 S 类之间的变化。

Griffith 将 II 型的 S 类菌加热到 100°C，灭活其致病性，而 II 型的 R 类菌已知无致病性，但 II 型的 S 类加热后、与 II 型的 R 类菌同时注射到老鼠时，可以致病、而且从患病死去的老鼠可获 II 型的 S 类菌。如果 II 型的 R 类与加热 100°C 处理的 I 型 S 类菌同时注射老鼠，结果无致病性，似乎同型的 S 类加热灭活后可以转化同型的 R 类。但 Griffith 用加热的 I 型 S 类菌与 II 型的 R 类菌共同注射老鼠时，可以致病，不过加热时的温度需要注意，60°C 可以、而 100°C 会灭活 I 型 S 类菌的转化活性。所以，在一定条件下不同抗原型的肺炎球菌，在加热失去致病性后，仍可将另一抗原型中无致病性的 R 类细菌转化为可致病的 S 类细菌。如果两类型都加热了，同时注射无致病性。Griffith 用 60°C 和 100°C 加热处理过其他型的 S 类肺炎球菌，然后检测其结果。他发现多数型的 S 类都能转变其他型的 R 类（如加热的 I 型 S 类变 II 型 R 类为 I 型 S 类、加热的 II 型 S 类变 I 型 R 类为 II 型 S 类、加热的 III 型 S 类变 I 型 R 类为 III 型 S 类、加热的 I 型 S 类变 IV 型的 R 类为 I 型 S 类），少数转化不行（如 IV 型的 S 不能变 I 型的 R 为 IV 型的 S，但能促进 I 型的 R 回复为 I 型的 S）。一型的 R 类加热后不能转化另一型的 R 类、也不能促进另一型的 R 类回复为 S 类。

Griffith 提出，S 类的菌含 S 物质，S 物质是蛋白质，它帮助制造细菌荚膜有抗原性的多糖。他当时认为，I 型的肺炎球菌同时有 I 型和 II 型的 S 抗原，只是 I 型的细菌含 I 型的 S 抗原多于 II 型的 S 抗原，而 R 类的 I 型细菌其 S 抗原大多

数变成了 R 结构，在加热后的 I 型 S 类细菌提供 S 物质的情况下，II 型 R 细菌获得 I 型的 S 抗原、获得致病性，也可因加热的 I 型 S 类菌含少量 II 型的 S 抗原，后者转 II 型 R 类菌荚膜导致 II 型 R 类菌回复为 II 型 S 类菌。

Griffith 的解释是从细菌抗原和致病性的角度，没有考虑遗传突变、回复突变和基因转导，但他一步一步从病例观察到提出和证明细菌转化，做出了关键的贡献。可惜，1941 年 Griffith 和同事一道逝于德国飞机轰炸。

### 3-6 Avery 实验室的主线：诊断、治疗和预防

Avery 实验室主要研究目的是在理解肺炎球菌的基础上，帮助诊断、治疗和预防细菌性肺炎，这些多数达到了、但时间不同，有些在几十年后他的学生的学生做到。

1877 年出生于加拿大的 Avery，十岁移民美国纽约，学过医但对研究更感兴趣，特别对危害人类的传染病（如肺结核）。1913 年，洛克菲勒医学研究所附属医院的院长 Rufus Cole（1872-1966）看重 Avery 在传染病方面的研究论文，请他加入洛克菲勒，Avery 从此精力集中于肺炎球菌。

在理论方面，1916 年 Dochez 和 Avery 提出“抗生长免疫”的概念（antiblastic immunity），认为抗体抑制肺炎球菌细胞内的酶活性，从而抑制代谢（Dochez and Avery，1916）。此理论当时就不为洛克菲勒内部其他学者（包括院长）所认同，一般认为抗血清导致细胞凝集后继发细菌生长减慢、代谢降低。Avery 这次错误使他以后更为谨慎。

在诊断方面，Avery 实验室从 1923 至 1934 年阐明细胞荚膜多糖抗原后，用细胞荚膜多糖抗原分型仍为今天追踪肺炎球菌的方法，到 2013 年知道有 93 型（Henriques-Normark and Tuomanen，2013），所以 Avery 的研究对诊断有帮助。

在治疗方面，Avery 到洛克菲勒附属医院后很快加入用抗血清治疗肺炎的研究，因此涉及分型，寻找针对不同型肺炎球菌的抗血清。抗血清的疗法在 1930 年代不如磺胺类药物的应用、1940 年代更不如青霉素的效果。在他们发现荚膜多糖对致病的重要性以后，再寻找降解多糖的方法，希望通过降解多糖、破坏荚膜来治疗肺炎。他于 1927 年在一次午餐时偶遇 Rutgers 大学毕业的研究生 René Dubos（1901-1982），Dubos 来自有土壤细菌专长的实验室、原导师为 Selman Waksman（1888-1973）。Avery 与 Dubos 一拍即合，觉得细菌在土壤里应该分解，所以在土壤里有降解多糖的分子，几年后他们确实找到了降解多糖的酶（Avery and Dubos，1931），而且可治疗动物的肺炎。不过，用蛋白质性质的酶作为治疗药物，不如小分子的抗生素，所以未得到广泛应用。

在预防方面，1914 年 Wright 等就曾为南非矿工制造疫苗以预防肺炎(Wright *et al.*, 1914)。真正得到公认有效的肺炎疫苗是 Avery 的学生 Colin MacLeod 有独立实验室后于 1945 年制造的针对多糖的治疗性抗血清（MacLeod *et al.*, 1945）。不巧正好抗生素被迅速推广，而青霉素成为治疗肺炎的标准药物，很多人以为无需疫苗预防，所以多糖的抗血清当时未得应用。但是，1964 年 MacLeod 的学生 Robert Austrian（1916-2007）发现细菌性肺炎仍有很高致死率（Austrian and Gold, 1964），重新提出研发和使用疫苗的必要性。Austrian 再度依据多糖抗原发明了多效价疫苗，成功地预防儿童肺炎（Austrian *et al.*, 1976），这一方法也是迄今肺炎疫苗的主流，所以在预防方面 Avery 本人播种而结果于他去世二十多年后他的学生的学生。

Avery 实验室有心栽花是诊断、治疗、预防，有些开了、有些没开、有些很晚开了。

## 3-7 Avery 实验室的支流：转化和遗传

Avery 实验室做转化实验初为无心插柳，最后成荫、成林…到今天是漫山遍野的万紫千红。

细菌转化实验是 Avery 实验室的支流。发现了现象最初不知其意义多大，断断续续地追踪，但最后意义远超出细菌性肺炎一种传染病，而是整个生物学的革命，也改观了传染病和非传染病的诊断、治疗和预防。

1916 年，Avery 实验室的 Stryker 就知道致病性和非致病性肺炎球菌的变化（Skryker，1916）。

1923 年 Griffith 第一次初步报道致病性和非致病性的肺炎球菌是 S 和 R 类后，洛克菲勒医院的 Reimann 于 1925 和 1927 年重复了 Griffith 的研究结果(Reimann，1925，1927)。

1928 年 Griffith 经典的转化实验发表前，德国柏林细菌研究所的 Neufeld 访问 Griffith 实验室得知其结果并进行了重复（Downie，1972），同年 Neufeld 发表验证文章（Neufeld and Levinthal, 1928）。1927 年 10 月离开纽约到洛克菲勒基金会支持的北平协和医学院内科任副教授的 Reimann，很快在北京继续肺炎研究，1928 年 10 月提交的文章于 1929 年发表（Reimann，1929）：他验证了 Griffith 的结果，而且用 Reimann 于 1925 年发现的不能自身在体外或体内变成 S 类的 I 型 R 类细菌，用 Griffith 的方法可以被转化为 S 类细菌，其抗原型取决于用来做转化的加热处理过的 S 类细菌的抗原型（I、II、III）。

Griffith 文章发表后，Avery 对其有怀疑。那几年他自己的注意力集中于寻找降解荚膜多糖的分子。

Avery 实验室从英属加拿大区来的博士后 Henry Dawson (1896-1945）相信英

国人 Griffith 的结果（Dubos，1976），并在体内重复验证（Dawson，1928，1930），但他试图在体外做转化实验未成功（Dawson，1930）。1930 年 Dawson 搬到哥伦比亚大学有独立实验室后，与协和医学院赴美国进修的谢和平（Richard Ho-Ping Sia）在体外细胞培养的条件下进行转化实验，也就是看加热后的一型 S 类的细菌能在体外共培养时转化另一型 R 类细菌（Dawson and Sia，1931；Sia and Dawson，1931）。但 Sia 与 Dawson 用肺炎球菌的提取物未能做成转化（Sia and Dawson，1931）。已知用来分型的抗原为多糖，而 R 类也有多糖的缺陷（才造成细胞荚膜缺陷），但把 S 类的多糖加到 R 类的细菌却不能将 R 类变成 S 类，所以转化物质不是多糖（Sia and Dawson，1931）。

1930 年 Dawson 离开洛克菲勒后，Avery 相信转换，鼓励 James Lionel Alloway 继续研究。1932 年，Alloway 发现用 I（或 III）型的 S 类肺炎球菌的提取物可将 II 型 R 类肺炎球菌转化为 I（或 III）型 S 类肺炎球菌（Alloway，1932），60°C 到 90°C 加热处理不能灭活提取物的转化活性，提取物的活性可以通过 Berkfeld 过滤器，而完整的细菌不能通过此类过滤器。Alloway 也指出用 S 的多糖不能转化 R（Alloway，1932）。1933 年，Alloway 继续摸索提取的条件，他发现不用 Dawson 的反复冻融方法而用脱氧胆酸钠 sodium deoxycholate 萃取能得到更多活性物质、用 5 比 1 的乙醇可以沉淀转化活性、用木炭可以吸附。他观察转化物质的化学性质，发现提取物无抗原性，所以肯定不是多糖抗原本身（Alloway，1933）。

### 3-8 生物化学途径

生物化学，既是科学，也是技术，而且迄今也是重要的技术。Avery 等 1930 到 1940 年代的探索为一例。

Alloway 与 1932 年离开 Avery 实验室后，Edward S Rogers 从 1932 到 1934

继续其研究。1934 年，加拿大的 Colin MacLeod（1909-1972）到美国加入 Avery 实验室，研究继续 Dawson 和 Alloway 的课题，从 S 类细菌的提取物中获得转化 R 类细菌活性物质。

MacLeod 改善了用于检测的细菌。起初 R 类细菌本身不稳定（容易变成同型的 S 类），而 S 类提取物的活性也不稳定。MacLeod 花了三年时间探索条件提高稳定性。1931 年谢和平与 Dawson 发现稳定性低的 R 类不仅易自身回复为同型的 S 类（I 型 R 类回复为 I 型 S 类）、且易被异型的 S 类（如 II 型的 S）转化（为 II 型的 S 类）（Sia and Dawson，1931）。不稳定的 R 类不很合适用来检验 S 的转化活性。MacLeod 用 II 型 S 类一株菌传 36 代后，于 1934 年获得 R36A 株菌，非常稳定、不易自身回复为同型 S 类，但却仍可以被加热的异型 S 转化为异型 S 类。用 R36A，MacLeod 重复了 Alloway 的实验，可以稳定地观察提取物的转化活性。

MacLeod 也探索了改进 S 类提取的方法。1937 年 MacLeod 用了 Sevag（1934）的方法用氯仿去除蛋白质。Alloway 当年发现病人的胸水有助于辅助转化反应，MacLeod 于 1936 和 1937 年花了相当时间找胸水中辅助因子，后来 McCarty 也找过，最后 Hotchkiss 不过是白蛋白，其作用也不特异（McCarty，1985）。从 1938 到 1940 的上半年，MacLeod 只零星做过转化方面的研究。他好几年都没发表文章，这时改研究磺胺对肺炎的作用等而发表文章。

Avery 患 Graves 病引起甲状腺亢进，1934 年做甲状腺切除术后短时休息，1935 年 Avery 可能就参与过转化实验。1940 年 10 月 MacLeod 和 Avery 两人合作做提取转化因子的实验（McCarty，1985）。以前每次用几升细菌做提取的起始，现在他们用 36 升，为了离心这么多有致病性的细菌，专门请人改装了密闭房间放置

离心机。他们于 1941 年 1 月 28 日第一次记录转化活性的粗提物中有少量 DNA（用二苯胺检测），而不仅有大量的 RNA（用 Biel 检测）。3 月 11 日 Avery 的记录表明，他试图先加热灭活肺炎球菌（这样先灭活了菌中可能降解转化物质的酶），再用脱氧胆酸钠破碎细胞制备提取，以便提高转化物质的产量。但 MacLeod 似乎有不同意见，3 月 18 日他们两人同时做实验，比较原来方法和 Avery 先加热的方法，觉得先加热有优点，以后都先加热（McCarty，1985）。Avery 给洛克菲勒的年度报告转化活性不为蛋白水解酶所降解、也不为 RNA 酶降解，当时有两个原因导致他们认为转化活性有可能是酯（ester）：几种含酯酶的生物制备可以灭活转化活性、能够抑制酯酶活性的氟化物业可以抑制那些生物制备对转化活性的灭活作用（Dubos，1976；McCarty，1985）。1941 年 7 月，MacLeod 离开 Avery 实验室到纽约大学任微生物系主任。

1941 年 9 月，Maclyn McCarty（1911-2005）加入 Avery 实验室。McCarty 在 Stanford 念本科、Hopkins 念医学院，对研究感兴趣。Avery 不给实验室的人指定课题，只是交谈后让各人自己决定，McCarty 与 Avery 和 MacCleod 交流后决定做转化因子（McCarty，1985）。他跟 Avery 学做细菌的实验常规，MacCleod 也教他。有些实验他和 Avery 一道做。McCarty 首先用 Dubos 提存的多糖降解酶再次证明转化因子本身不是多糖。以前还没有完全放弃多糖可能有利于转化活性，这次实验后 McCarty 和 Avery 决定改变长细菌的方法降低多糖的含量，他们以前长细菌是用较多的葡萄糖，现在去除这种多余葡萄糖，结果不仅多糖减少，而且转化活性增加。McCarty 还决定在加热处理细菌后、胆酸提取前再多洗几次粘在细菌表面的多糖，结果也提高了转化活性的产量。1941 年 12 月的这些改进后，1942 年 1 月，McCarty 发现乙醇处理转化提取物时出现纤维状物质。在 Avery

实验室两层楼上，Alfred Mirsky 碰巧发现自己研究的物质与染色体有关，Mirsky 与哥伦比亚大学的 Pollister 合作得到分离染色体 DNA 的方法：先用盐处理细胞得到细胞核，再用盐得核蛋白复合体（nucleoprotein），用氯仿去除蛋白质后，加乙醇沉淀 DNA，而加乙醇时可以用木棒搅动溶液，纤维状的物质会缠绕木棒，得到很纯的 DNA。1942 年 3 月 McCarty 试了这一方法，确实得到可以用二苯胺法检测到的 DNA、并有转化活性。McCarty 和 Avery 还与洛克菲勒的生物物理学家 Alexandre Rothen 合作使用当时很新的超级离心机，发现转化物质的分子量很大，当时只有 DNA 可以类似。进一步用用超速离心机制备的物质含 DNA 和转化活性。在有两种结果提示转化活性是 DNA 的情况下，他们进一步用酶处理来检验 DNA 与转化活性的相关性。他们（和以前 MacLeod）知道多种酶的制备可以抑制转化活性，他们检验是否这些制备可以降解当时标准的 DNA（从胸腺提取的 DNA），结果看到相关性。所以，到 1942 年夏天多方面结果指向转移因子是 DNA。7 月，他们与 Mirsky 合作，由 McCarty 提供大量肺炎球菌，Mirsky 用自己提取核蛋白的方法，获得后由 McCarty 检测转化活性，结果确实有。这时并未去除核蛋白的蛋白质部分，但 McCarty 觉得用完全不同于他提取转化活性的方法得到的含 DNA 的核蛋白复合体也能有转化活性，更支持 DNA 可能是转化物质。

摸索不同条件后，他们综合为提取方法：培养细菌离心后置 65$^{o}$C 加热，用生理盐水洗（洗掉多糖），用脱氧胆酸处理，用乙醇沉淀获得纤维状物质（包括还存在的多糖和 DNA），用 Sevag 的氯仿去除蛋白质，加 Dubos 的多糖酶（SIII），降解多糖，直到用多糖抗体检测不出多糖，再次用 Sevag 方法氯仿去多糖酶（也是蛋白质），最后再用乙醇沉淀，这是被乙醇可以沉淀的多糖已经没有了，只剩

DNA 可以被乙醇沉淀，逐渐加入乙醇导致沉淀的 DNA 形成纤维状物质用搅棒收集。一般他们用 200 升细菌，可以得到 45 毫克的终产物。

McCarty 和 Avery 的实验常出各种问题，需要坚持、改进，解决大小问题。他们每天早上两人一道看结果，Avery 的表情是又期待又怕出问题，发出“失望是我每天的面包”的叹息。这恐怕是多数实验科学研究者的日常生活。

1942 年 11 月和 12 月，他们得到的转化物质，元素分析其氮和磷含量都相同于 DNA，并于 1943 年 2 月和 3 月重复。1943 年 4 月 Avery 给洛克菲勒科学顾问委员会的报告中写道：转化因子--核酸—可比为基因（gene），它导致合成的终产物多糖为基因产物（gene product）…转化一旦发生可以不断传代的事实支持对转化的遗传理解（McCarty, 1985）。1942 年 10 月 Avery 到了 65 岁的退休年龄，预计 1943 年 6 月退休搬到田纳西州他弟弟 Roy Avery 附近，1943 年 5 月，Avery 写信给弟弟说明为什么不马上搬，讲述了转化因子的工作：谁能猜到是 DNA…是可预计和遗传的变化—遗传学家的梦想…听起来是病毒，可能是基因(Dubos, 1976)。

1943 年 5 月他们继续实验，得到产物的活性很强（$3x10^{-9}$ 克有作用），请物理化学的同事 Theodore Shedlovsky 做电泳实验，他们提取的转化物质只有一条带，且电泳后还有活性，进一步支持提纯的 DNA 分子在起作用。其后，Avery 还不放心，他们专门咨询了几位生化学家，其中一次是 Avery、MacLeod、McCarty 三人一同到当时还在纽约城外 Princeton 的洛克菲勒研究所分部见蛋白质化学专家 John Nothrop 和 Wendell Stanley（1946 年诺贝尔化学奖得主）。只在德国移民到洛克菲勒工作的蛋白质化学家 Max Bergmann（1886-1944）处得到有用的信息：所谓无论哪里来的 DNA 都一样的说法是胡说，如果它们是大的多聚物，完全可

能有无穷的组合，导致化学结构不同而元素组成相同。McCarty 于 7 月补了实验，证明被转化的细菌中可以提取到转化因子（McCarty，1985）。

Avery 在缅因州度假写文章，McCarty 写实验方法，MacLeod 写前期的结果，Avery 提出是否按资历、与课题相关时间排作者顺序也不一定合适，可能按字母顺序，McCarty 说两种方法的结果都是一样的：Avery、MacLeod、McCarty，同意。1943 年 10 月 Avery 向洛克菲勒研究人员宣读研究结果，当时只有鼓掌、无人提问。11 月 1 日 Avery 把稿件交给洛克菲勒主板的《实验生物学杂志》的主编 Peyton Rous（1879-1970，1911 年发现病毒致癌，1966 年诺贝尔医学奖），Rous 对稿件有修改建议，包括要求删除“核酸的重要性能否与氨基酸链相媲美成为一个问题”。1944 年 2 月 1 日文章出版。

### 3-9 转化因子（Transforming Principle）

1944 年 Avery、MacLeod、McCarty 共同发表论文（Avery, MacLeod and McCarty，1944），一般认为与 Watson 和 Crick（1953）并列为二十世纪生物学最重要的文章。

Avery-MacLeod-McCarty 开篇高屋建瓴，完全不同于前面一系列限于肺炎球菌分型的文章，而是将问题提高到遗传的分子机理层面：首先指出很多生物学家试图用化学的方式在高等生物诱导特异、可预见、能够遗传的变化。其次认为微生物中可以实验诱导、重复发生的、可遗传的特殊变化最好的例子是肺炎球菌型的转化实验。其后他们简介 Griffith 的实验结果，以及 Dawson、Alloway 等的进一步工作。Avery-MacLeod-McCarty 的研究目的是找有转化活性的因子（principle），并初步确定其化学性质、化学分子的类型。

达到宏大的目标需要能解决问题的具体方法。用来获得提取物的是 III 型 S

类菌株（A66），与被转化的 II 型 R 类有很大的形态和大小差别。转化实验用的培养液，MacLeod 用炭可去除不稳定性。转化实验仍需血清，以前谢与 Dawson 认为血清提供抗 R 的抗体，Avery 等认为与抗体无关，但仍需血清的某种其他成分，因血清含灭活转化活性的酶，需将血清加热到 60°C，灭活此酶。用于检测转化活力的细菌，需不易自身转变、而同时对转化活性有反应，这就是 MacLeod 用 II 型 S 传 36 代后获得的 R36A 菌株。他们注意到即使是 R36A 还可衍生不同的菌株，有些可被转化、有些不能，而 R 菌破损释放的酶能降解 S 菌的转化活性，所以需选生长良好的 R 以免检测的不稳定。他们采用 Sevag (1934)发明的方法用氯仿去除蛋白质、接着用乙醇多次沉淀，用 Avery 实验室发现的降解多糖的酶去除多糖，再逐滴用乙醇达到临界浓度沉淀活性物质，得到粘性的纤维，用玻璃棒搅出来再溶解。乙醇沉淀 DNA 的方法是洛克菲勒生化学家 Alfred Mirsky 告诉 McCarty（Mirsky and Pollister, 1946；Darnell，2011）。

提取的活性物质在生理盐水中很稳定可保存数月、但在水中不稳定，65°C 一小时不能灭活，用两种检测蛋白质的方法（双缩脲和 Millon 检测）结果显隐性，用 DNA 的检测方法（Dische 氏二苯胺反应）显强阳性，RNA 检测方法（Bial 氏地衣酚反应）显弱阳性，但他们用同样浓度的提取的胸腺 DNA 看到用地衣酚反应也显弱阳性。反复用乙醇和乙醚提取不降低活性，推测活性分子不是脂肪。

他们对四批提取的转化物质进行了元素含量检测，含 C 约 34%、H 约 3.8%、N 约 15%、P 约 9%，都接近纯化的胸腺 DNA 中元素的含量（34%、3.2%、15%、9%）。N/P 比例为 1.58 到 1.75，也接近 DNA 预计值 1.69。

他们从洛克菲勒的酶专家 Northrop 和 Kunitz 获结晶纯的胰蛋白酶、糜蛋白酶和 RNA 酶，它们都不能灭活转化因子，说明转化活性非 RNA、也非这些蛋白

酶能降解的蛋白质。

他们从几种动物组织制备粗提物，a 狗小肠粘膜、b 兔骨磷酸酶、c 猪肾磷酸酶、d 肺炎球菌自溶物、e 狗和兔血清。在酶活性方面，这些粗提物除 d 外都有磷酸酶活性、除 c 外都有甘油三丁酸酯酶活性、除 b 和 c 外都有 DNA 酶活性（检测 DNA 酶活性的 DNA 由洛克菲勒的 Mirsky 提供），而只有 a、d 和 e 能够灭活转化物质，比较磷酸酶、酯酶和 DNA 酶活性的相关性倾向于支持 DNA 是转化因子。

他们用不同温度和时间处理狗和兔血清，观察到热灭活 DNA 酶活性负相关于这些制备对转化因子的灭活。而温度灭活血清中甘油三丁酸酯酶的活性与转化活性无关。

他们试了一些化合物能否抑制灭活转化因子的酶，发现氟化钠有显著作用，它可以抑制所有已知来源（如肺炎球菌、狗小肠粘膜、胰腺、血清）的 DNA 酶的活性，也抑制这些来源 DNA 酶制备物对转化因子的灭活，进一步支持 DNA 与转移因子的相关性。

转化因子本身对 III 型抗血清无反应，不是 III 型的抗原本身，也就不是荚膜多糖。

他们请生物物理学家分析，发现在超速离心中，转化物质显示均质性和非对称性，分子量约 50 万。电泳行为也显示类似核酸的一种分子。UV 光谱显示吸收峰在 260nm，也与核酸一致。

转化因子活性很强：2.25 毫升溶液含 0.003 微克仍有活性，六亿分之一的比浓度。

在讨论中，他们说从 III 型肺炎球菌分离了 DNA 组分可以将 II 型衍生的无荚

膜 R 类菌转化为完整荚膜的 III 型菌。肺炎球菌有 RNA 是 1938 年洛克菲勒的`科学家所发现（Thompson and Dubos，1938），而 1944 年前尚无人报道肺炎球菌的 DNA。他们强调转化因子的化学性质不同于其导致产生的荚膜多糖。他们指出“本文所呈现的资料强烈提示核酸，至少是脱氧类型的，具有转化因子选择性作用所证明的不同特异性”。他们也知道细菌一旦被转化后，获得的性状可以传代，转化带来的变化“可预计、有型的特异性、可遗传”。

对转化的机理，Avery-MacLeod-McCarty 讨论了三种，其中第二种是遗传学家 Dobzhansky（1941）提到的转化可能是遗传突变（“如果转化被描述为遗传突变—很难避免如此描述—我们就在面对特别处理导致特异突变的真实例子”），Avery-MacLeod-McCarty 认为这是比拟转化因子是基因。

他们承认还可能有其他物质吸附在核酸上起作用，但如果确实能证明就是核酸起转化作用，那么核酸应该具有尚未确定化学基础的生物学特异性。

全文的结论：出示的证据支持脱氧类的核酸是 III 型肺炎球菌转化因子的根本单位（the evidence presented supports the belief that a nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type III）。

Avery-MacLeod-McCarty（1944）的文章是 1943 年 11 月提交，措辞含蓄可能是因为他在提出多糖抗原时经历过争议有经验。而在 1943 年 5 月 26 日 Avery 在给自己弟弟的信中，他清楚地说明转化是可以遗传、可以预计的变化，可能是基因（Dubos，1976）。

1945 年，英国神经生理学家、1936 年诺贝尔奖得主 Henry Dale（1875-1968）在给 Avery 发英国皇家学会的 Copley 奖时，提到 Avery 发现了基因（Dale，1946），而诺贝尔奖委员会受其研究核酸的成员 Hammarsten 影响直到 1952 年以前都不

认同 DNA 是遗传的物质基础（Reichard，2002）。

1943 年 Avery 到了强制性退休年龄，但二战期间美国怕出现战时传染病而请微生物学家为国家工作，洛克菲勒医学研究所也给 Avery 荣誉研究员、实际继续主持实验室。终生未娶的 Avery 于 1947 年离开纽约去田纳西州，与任教 Vanderbilt 大学微生物系的弟弟 Roy Avery 比邻而居过退休生活，1955 年去世。

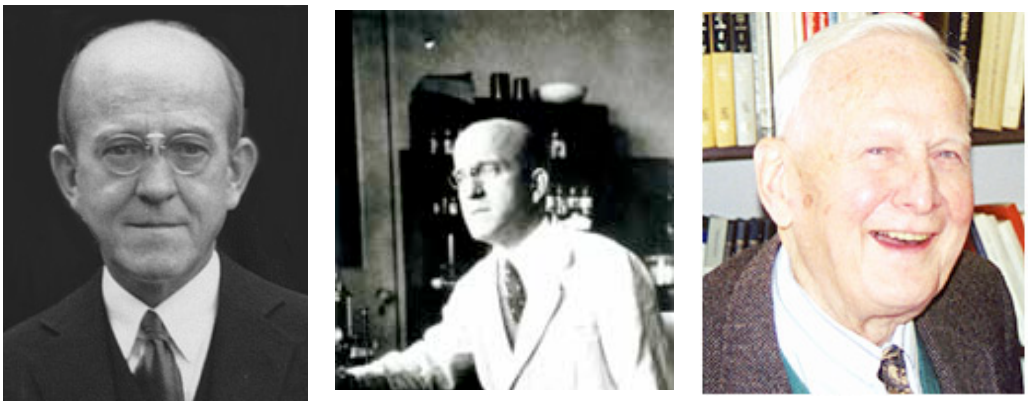


图 5. Avery （1877-1955）；MacLeod （1909-1972）；McCarty （1911-2005）

### 3-10 对新概念的反应

当时的科学家如何理解 1944 年 Avery、MacLeod、McCarty 的文章？知道 Avery-MacLeod-McCarty 工作的科学家面临三种很重要的的选择：不理不睬、检测真伪、走下一步。

1949 年，McCarty 应邀到 Johns Hopkins 大学医学院学术演讲，正好安排在一位晕船研究者后，大批听众在听完晕船报告后离开，只有 35 人耐心听 McCarty 讲 DNA 与细菌转化（McCarty, 1985）。1950 年，为遗传学五十周年举行纪念的学术活动中，26 位撰稿人只有一位提到 Avery 的 DNA 工作（Dunn， 1951），而这位还是 Avery 的同事 Mirsky，他并不认为 Avery 的工作证明了遗传物质是 DNA 而非蛋白质。

3-10-1 很难排除 DNA 之外完全无蛋白质

十九世纪核酸的生物化学权威是 Miescher 和 Kossel，二十世纪 1940 年以前核酸的生化权威是洛克菲勒的 Levene，而 1940 和 1950 年代核酸生化的权威为 Alfred Mirsky（1900-1974）。Mirsky 于 1927 年加入洛克菲勒医学研究所前期研究蛋白质（特别是血红蛋白），1940 年代后主要研究细胞核的核酸和蛋白质。二十世纪上半叶两位核酸生化权威 Levine 和 Mirsky 皆低估核酸的重要性。

Mirsky 和哥伦比亚大学的 Arthus Pollister 主要依赖不同浓度的 NaCl，从精子、肝、胰、肾等将细胞核中核酸和蛋白质的复合体（时称“核蛋白”nucleoprotein）与细胞质分开，获纤维状物质（Mirsky and Pollister，1942）。他们还能分开复合体的核酸和蛋白。Mirsky 改进方法后不仅可从动植物等真核细胞获得细胞核的核蛋白，且可从显微镜看不到细胞核及染色体的原核生物（细菌）中提取核蛋白，他们提出所有细胞都有 DNA 和组蛋白，在组蛋白以外染色体还含其他蛋白质（Mirsky and Pollister, 1946）。他们用来提取核酸和蛋白质的细菌正是 Avery 给他们的 III 型肺炎球菌，并提到 Avery-MacLeod-McCarty 提取转化因子的方法部分参考了 Mirsky 和 Pollister 方法（Mirsky and Pollister, 1946），与 McCarty 后来的回顾一致（Darnell, 2011）。Mirsky 和 Pollister（1946）指出核蛋白的纤维状是因多聚体的 DNA，而非 1937 年诺贝尔奖得主 Albert Szent-Györgyi（1893-1986）等误认为与肌球蛋白（myosin）相似。他们也用提取的核蛋白（当时他们还曾称为 chromosin）验证了 Avery-MacLeod-McCarty 的转化实验，但他们指出很难获得完全不含蛋白质的纯 DNA，而当时的方法无法检测出低于 2%的蛋白质，所以不能断定转化活性是 DNA、还是蛋白质，他们认为还需一步一步去除蛋白质、同时检测转化活性（Mirsky and Pollister, 1946）。他们曾认为可以用氯仿-辛醇反

复 6、7 次去除蛋白质、再用乙醇沉淀后获纯化的 DNA（Mirsky and Pollister, 1946），但他们未进一步用自己提出的方法来验证转化因子是核酸还是蛋白质。Mirsky 多次较强烈公开表示反对只有 DNA 而无蛋白质参与转化。

1947 年，Mirsky 在提取染色体后确定 DNA 占其质量的 37%和蛋白占 59%（Mirsky and Ris, 1947）。他们继而发现同一生物的不同体细胞中 DNA 含量相同，而体细胞 DNA 含量是精子 DNA 的两倍，因为体细胞是二倍体有两套染色体、而精子是单倍体，所以 DNA 含量与染色体数量相关，Mirsky 也相信 DNA 可能是遗传物质的一部分，但并不表明基因只有核酸没有其他（Mirsky and Ris, 1949, 1951）。Mirsky 至 1951 年仍不信仅由 DNA 而无蛋白质携带遗传信息（Mirsky，1951）。

Mirsky 与 Avery 的关系因为前者在私下和公开批评 Avery-MacLeod-McCarty 而恶化，Avery 还在洛克菲勒工作的最后几年，他们之间断绝了直接讨论和交流（McCarty，1985）。

3-10-2 纯 DNA 的转化活性

Avery-MacLeod-McCarty（1944）认为转化因子是 DNA 非蛋白质的证据之一是 DNA 酶可以降解转化因子，而蛋白酶、RNA 酶不能。但是，当时用的 DNA 酶并非纯化的 DNA 酶，而是很粗糙的制备，含很多其他的酶，Avery-MacLeod-McCarty 只比较了这些制备物含磷酸酶、酯酶和 DNA 酶的相对分布，所以他们也承认有关转化因子的酶学证据是间接的（McCarty and Avery, 1946a）。实际上，DNA 酶不过是 1940 年才由美国国立健康研究院癌症研究所的 Jesse Greenstein 和 Wendell Jenrette 所发现、1943 年命名，要到 1948 年洛克菲勒的 Kunitz 才能获结晶纯的 DNA 酶（Kunitz，1948）。1944 年

Avery-MacLeod-McCarty 文章发表时，全世界谁也没有纯化的 DNA 酶（DNase）。

为解决这一问题，1942 年 McCarty 就请技术员 William La Rosa 纯化 DNase，进展不大，1943 年 McCarty 自己开始从牛胰纯化 DNase，其降解 DNA 的活性可以从处理 DNA 后，DNA 的粘稠度来检测。这样制备的 DNA 酶可以降解转化活性，但结果比较初步，没有放到 1944 年发表的文章中（McCarty，1985）。

1944 年，McCarty 到当时在 Princeton 的洛克菲勒研究所的分部，跟 Kunitz 学习分离结晶蛋白质。Kunitz 跟 Northrop 成了专家，不仅结晶了 RNA 酶，还获得了结晶纯的好几个酶。McCarty 学会了他的技术，Kunitz 能结晶的五个酶，McCarty 回到纽约的分部也都能结晶，只有 DNA 酶虽然纯化度提高了、但不能结晶。1946 年 McCarty 发表论文，报道获的相当纯化 DNA 酶、活性很高，不含 RNA 酶、酯酶、磷酸酶、但含很低的蛋白水解酶活性（McCarty, 1946a）。镁离子可激活、柠檬酸可抑制 DNA 酶，特异的抗血清也可抑制 DNA 酶（McCarty, 1946a）。用经过一定纯化制备的 DNA 酶，他们再检验转化因子是否 DNA（McCarty and Avery, 1946a）。这次，他们也观察了相关性。镁离子激活 DNA 酶活性，但镁的作用可以被柠檬酸抑制，而锰离子也激活 DNA 酶活性，但锰的作用不被柠檬酸抑制。而这些作用都与柠檬酸能否抑制镁离子和锰离子对转化活性的作用相关。对 DNA 酶制备物还含微量蛋白水解酶活性的问题，他们用稀释的方法予以排除。制备物在含蛋白质每毫升 0.2 毫克以上时可以检测到蛋白水解酶的活性，但他们稀释到蛋白质浓度低于每毫升 0.01 微克、直至每毫升含 0.0025（甚至 0.00125）微克时，还有 DNA 酶的活性和降解转化活性的作用，而这时无蛋白水解酶活性（需要浓度提高十万倍才有），这样的结果应该很难否定转化因子是 DNA 的可能性。

发现柠檬酸对 DNA 酶的抑制作用后，McCarty 和 Avery 再重新提取 III 型 S 类菌的转化因子，在提取过程中加柠檬酸，提高了转化因子产量五倍（McCarty and Avery, 1946b）。他们从 II 型和 VI 型肺炎球菌也找到了转化因子，对 II 型转化因子的分析也支持是 DNA。

1946 年 Rollin Hotchkiss（1911-2004）加入转化因子研究。1948 年 Kunitz 获得结晶纯的 DNase 后，Hotchkiss 证明它可以灭活转化因子（McCarty，1985）。至 1949 年，Hotchkiss 可以做到提取的转化物质中蛋白质含量低于 0.02%却仍有转化活性，而且在纯化过程中虽然蛋白质含量越来越低、但转化活性不降低，如果转化因子还是蛋白质，那就非常不同于其他蛋白质（Hotchkiss, 1952）。

### 3-10-3　核酸的特异性和信息量

Levene 的“四核苷酸假说”导致误解：核酸单调无信息含量。

核酸生化权威如此，而在二战前后都研究核酸生物物理的英国科学家 William Astbury（1898-1961）也认同四核苷酸假说（Astbury，1947）。

哥伦比亚大学的旅美奥地利犹太生物化学家 Erwin Chargaff（1905-2002）受 Avery-MacLeod-McCarty（1944)结果的激动，从脂类研究转向核酸研究，用生化方法检测不同组织、细胞来源的 DNA 是否含等量的四种核苷酸。他实验室将 1944 年发明的纸层析方法用于分开核苷酸、用紫外线分光光度仪检测核酸，提高了灵敏性与可靠性（Vischer and Chargaff, 1947, 1948）。几年内，他们很快发现 A:G:C:T 不等于 1:1:1:1，推翻了单调重复的四核苷酸假说、支持 Avery-MacLeod-McCarty 提出的核酸可能具有化学特异性（Chargaff *et al.*, 1949; Vischer *et al.*, 1949）。有了 DNA 酶以后，他们将牛胰 DNA 降解为多个片段，发现不同片段含 A、G、C、T 量不同，从而提出 DNA 链中核苷酸有非单调的排列（Zamenhof and Chargaff,

1950; Tamm *et al*., 1953)。

Chargaff 最初只关注不同来源 DNA 中核苷酸是否不同，其实他们 1949 年的文章中的资料就还有更多信息，如 A:T=1:1、G:C=1:1（Chargaff *et al*., 1949; Vischer *et al*., 1949），但他们当时没有注意到。1950 年，Chargaff 写综述总结自己实验室的工作时，意识到多种来源的 DNA 中常常 A:T=1:1、G:C=1:1（Chargaff, 1950），但他不清楚是巧合还是真的规律，只提请注意。即使 1951 年他们在精子再度观察到这样的比例，也不敢断定是否巧合（Chargaff *et al*., 1951）。这一比例后人称为 Chargaff 规则，为 Watson 和 Crick 的 DNA 双螺旋两条链的碱基配对提供了伏笔。Chargaff 还发现同种生物的 DNA 相同，而不同生物之间不同，说明 DNA 有生物特异性，有时这也被称为 Chargaff 规则之一。他还注意到同一生物的 RNA 在不同器官不同，他提出可能这些不同 RNA 参与器官分化（Chargaff，1950）。

### 3-10-4 转化的普遍性及其与遗传的关系

认同 Avery-MacLeod-McCarty（1944）工作有很大意义的科学家也认为当时还不能完全接受 DNA 是遗传物质。因为即使转移因子只含 DNA 而无蛋白质，Avery-MacLeod-McCarty（1944）的结果还可以有多种解释， 1958 年的诺贝尔奖获得者在 1956 年总结为：1）转化因子不是遗传物质，而是一种突变剂；2）多糖合成的自身催化剂；3）能够诱导宿主细胞荚膜合成反应的细菌病毒；4）细胞质基因或形态发生源；5）未进入细胞起作用；6）细菌遗传机构中能检测的一部分；7）特有的机制、无普遍性（Lederberg, 1956)。

1946 年，McCarty 在美国细菌学会获奖时已经说明转化因子的作用是可遗传的，被转化的细菌不仅性状传代，而且从后代提取到的转化活性可以高于最初用的转化活性，说明转化因子不仅仅能诱导荚膜多糖合成，而且能在细菌中自我复

制，这些特性既与基因相似、也与病毒相似（McCarty, 1946b），所以 Lederberg 在 1956 年都没意识到他提的 7 点有很多可为 1946 年 McCarty 已知的结果所排除，而 Lederberg 还属于特别推崇 Avery-MacLeod-McCarty 者之一。

对于普遍性的问题，1947 年法国的 André Boivin 报道可用来自一种抗原性的大肠杆菌的 DNA 转化另一大肠杆菌的抗原性，他称为定向突变（directed mutation）（Boivin, 1947）；1949 年 Austrian 和 MacLeod 在肺炎球菌可同时做到三个性状的转化（Austrian and MacLeod, 1949）；1951 年 Hotchkiss 发现从青霉素抵抗的细菌可得 DNA，将青霉素抗药性转给其他细菌（Hotchkiss, 1951）；1951 年美国哥伦比亚大学儿科的 Hattie Alexander 与 Grace Leidy 发现流感嗜血杆菌的 R 类也可以被 S 类的 DNA 所转化，而且这时他们用了结晶纯的 DNA 酶，证明转移因子是 DNA（Alexander and Leidy, 1951）。他们 1953 年发现流感嗜血杆菌的链霉素抗药性可通过 DNA 转化到原来对链霉素敏感的流感杆菌，起转化作用的 DNA 可被结晶纯的 DNA 酶所降解，他们还发现脑膜炎球菌分型相关的性状可通过 DNA 转化（Alexander and Redman, 1953）。

DNA 可在多种细菌之间转化多种可以检测的性状，转化后的性状都可在代间遗传，从被转化的细菌的后代获得的 DNA 本身也有转化活性，证明 DNA 的作用之普遍性和可遗传性。

### 3-10-5 Hershey-Chase 实验

应该说 1946 年 McCarty 和 Avery 文章后，DNA 是转化因子的可能性非常大，而 Hotchkiss（1952）纯化的转化因子蛋白质含量低于 0.02%，1951 年 Alexander 与 Leidy 用结晶纯的 DNA 酶可降解流感杆菌的转化活性就基本证明了 DNA 的转化因子。但如果要一直质疑，还可以说降解了 DNA 链后导致上面附着的微量

蛋白质活性不稳定。要完全证明只有 DNA 是转化因子，需要确定某些基因的核苷酸序列、然后化学合成同样的序列、再用来转基因，而当时在知识上和技术上都不可能做到，只能依靠不断增强的外围证据。

1950 年代影响很大的工作是 Hershey-Chase 实验（Hershey and Chase, 1952）。Alfred Hershey（1908-1997）研究细菌的病毒（噬菌体），1950 年到冷泉港实验室工作，Martha Chase（1927-2003）为其助手。噬菌体可以感染细菌，并在细胞内复制、细菌间传代。噬菌体外壳为蛋白质、内含 DNA。Hershey 和 Chase 用放射性同位素标记核酸和蛋白质以观察当细胞外的噬菌体感染细菌时，核酸和蛋白质的行为。磷 32 用于追踪 DNA（所有 DNA 都含磷）、硫 35 用于追踪蛋白质（有些氨基酸含硫）。在噬菌体感染细菌几分钟后，Hershey 和 Chase 摇动培养将细菌与细胞外的噬菌体分开，再检测磷和硫分别多少进入细胞、多少留在胞外，结果发现 30%的磷 32 留在细胞外、80%的硫 35 留在细胞外；代间观察发现，30%的磷 32 传代、少于 1%的硫 35 传代。这一实验被认为提供了很强的证据表明 DNA 是遗传物质、蛋白质不是。如果要比较，当然这些结果在纯度上远不如 1946 年 MaCarty 和 Avery 已经达到的程度，更不如 Hotchkiss（1952）的程度。而且因为有些蛋白没有含硫的氨基酸，并非所有的蛋白质都能被硫 35 所标记。可能因为 Hershey 获诺贝尔奖的缘故，来自这一实验的图曾长年被很多教科书拷贝而未意识到其结论并非很好的证据。

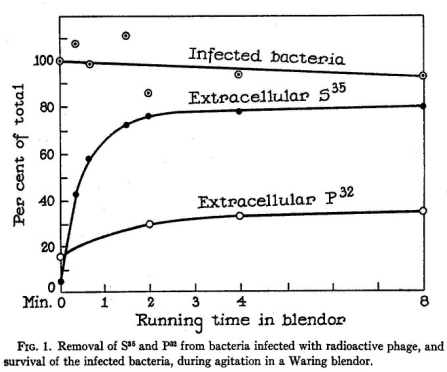



FIG. 1. Removal of $S^{35}$ and $P^{32}$ from bacteria infected with radioactive phage, and survival of the infected bacteria, during agitation in a Waring blendor.

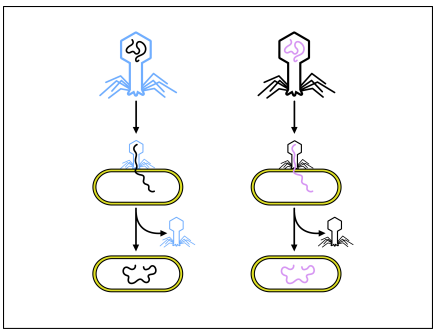


图 6. Hershey-Chase 实验。左：结果；右：示意图 （左为 S35 标记蛋白质，右为 P32 标记核酸）

3-10-6 细菌遗传学

Avery-MacLeod-McCarty 后续研究的一个方面是以上验证 DNA 是转化因子、并推广其意义，另一方面是刺激以后的发展。

美国遗传学家 Joshua Lederberg（1925-2008）于 1945 年念研究生的期间读到 Avery-MacLeod-McCarty，他记录 1945 年 1 月 20 日一个晚上读文章后兴奋状态，认为意义“无限”(unlimited in its implications)、有突变特征、可以复制。他在 1994 年回顾 1945 年时认为 Avery-MacLeod-McCarty 可以声称：a）肺炎球菌有可遗传的性状，如血清学特异的多糖细胞荚膜，与致病性有关，并可在老鼠或血清中被选择；b）这些性状的遗传原基可通过无细胞的提取物在菌株之间转移，称为转化；c）转化因子的化学结构是 DNA，不是蛋白质或其他大分子。如果这三点成立，那么就带来激进的概念：d）细菌有与高等动物一样的基因；e）基因是 DNA；f）细菌可用于遗传学研究。

1943 年，意大利裔美国犹太生物学家 Salvador Luria（1912-1991）和德裔美国物理学家 Max Delbrück（1906-1981）借助数学模型分析细菌对噬菌体抵抗性的统计规律，证明细菌可以自发出现遗传突变（Luria and Delbrück, 1943）。美国

Stanford 大学的 Edward Tatum（1909-1975）1941 年与遗传学家 George Beadle（1903-1989）通过遗传突变研究粗糙链胞霉（Neurospora）的生化反应（Beadle and Tatum, 1941）、最终提出一个基因一个酶的概念。Tatum 于 1944 年和 1945 年研究了细菌（K12 大肠杆菌）的突变。

还在哥伦比亚大学念医学院的 Lederberg 从本科阶段就参加科学研究，其指导老师曾跟随 Tatum 用粗糙链胞霉。1945 年 1 月，Lederberg 从一位研究生那里获 Avery-MacLeod-McCarty 全文，读后兴奋不已。他想用链胞霉研究转化，结果发现他们最初用的链胞霉突变种很容易自发回复，没做成他预计的实验，但发表了他第一篇论文。他再转向研究细菌是否有“性”。他的老师听说 Tatum 要来东海岸到耶鲁工作，建议他到 Tatum 实验室一道研究。Lederberg 给 Tatum 写信提出他的研究目的：细菌的性重组（sexual recombination）。他们在耶鲁很快出了结果，发现细菌的性生活：一个细菌将信息给另一细菌（Lederberg and Tatum, 1946; Lederberg, 1947）。细菌遗传学在 1950 和 1960 年代为遗传学的核心，其所用的细菌、发现的质粒（Lederberg，1952）等也成为分子生物学和 1970 年代诞生的生物技术产业的重要工具。

3-10-7 解析 DNA 三维结构

美国生物学家 James D Watson（1928-）是印第安纳大学 Luria 的研究生，他于 1950 年毕业，学了噬菌体和遗传学，他非常接受 Avery-MacLeod-McCarty 的观点，认为 DNA 就是遗传物质。Watson 到英国剑桥大学以物理见长的、麦克斯韦曾为第一任主任的卡文迪许实验室做博士后期间，不愿按导师 John Kendrew（1917-1997）安排研究蛋白质或病毒的结构，而与 Max Perutz（1914-2002）的研究生 Francis Crick（1916-2004）热衷讨论 DNA 的结构（Watson, 1968）。Watson

和 Crick 与伦敦国王学院用 X 线衍射 DNA 的 Maurice Wilkins（1916-2004）和 Rosalind Franklin（1920-1958）有很多讨论，最初他们提出的三螺旋模型被 Franklin 指出有明显错误，其后还得益于看到 Franklin 拍摄的一张衍射图片，提出 DNA 双螺旋模型。Franklin 也独立地提出了 DNA 双螺旋模型。1953 年 4 月 25 日同一期 *Nature* 刊登（Watson and Crick, 1953a; Wilkins *et al*., 1953; Franklin and Gosling, 1953）。只有 Watson 和 Crick（1953）提出了碱基配对，也就解释了 A/T、G/C 比例为何为一，很快他们提出 DNA 复制的机理（Watson and Crick, 1953b）。双螺旋结构很漂亮，而生物学意义重要的是碱基配对。物理学在分子生物学的建立过程起了很大作用。

注 1：Avery-MacLeod-McCarty（1944）和 Watson and Crick（1953）是二十世纪最重要的两项生物学研究。如果认为首先需要确定 DNA 是遗传物质，其后分析 DNA 结构才重要，也可以说 Avery-MacLeod-McCarty 更重要。

注 2：英国生物化学家 Alexander R. Todd (1907-1997) 获 1957 年诺贝尔化学奖，其中部分工作是核酸。他于 1952 年提出的 DNA 化学结构与 Levene 和 Tipson 于 1935 年提出的相同，诺贝尔奖颁奖时对他研究核酸“十五年”工作的赞扬都在 Levene 基本做完了大部分核酸化学的工作之后 (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1957/press.html)。

注 3：1944 年，Avery 已 67 岁，属很少见的在年龄很大的时候做出重要工作的科学家。MacLeod 只有 35 岁，McCarty 只有 33 岁。MacLeod 一直是“少年得志”类，15 岁高中毕业，23 岁毕业于 McGill 大学医学院，到纽约大学任微生物系主任时仅 32 岁。

注 4：据 Hammarsten 的学生 Reichard（2002）在研读解密的文件后介绍，1932

年至1946年几乎每年Avery都因发现细菌多糖的抗原性而被提名诺贝尔奖。1946年后提名开始提到他的转化工作，但当时诺贝尔医学奖委员会懂核酸的关键成员Hammarsten 虽首先提议委员会考虑这一工作，但他每次都认为不行，前几年是考虑到提取物易污染、难排除蛋白质起转化作用的可能性，等到 1952 年 Hershey 和 Chase 文章、1953 年 Watson 和 Crick 文章后，他和其他成员相信 DNA 是转化因子，Hammarsten 的评估报告认为 DNA 是转化因子，但其作用机理不明、所以得奖还早。1953 年诺奖给三羧酸循环的发现者 Krebs 和辅酶 A 的发现者 Lipmann、1954 年奖给发现脊髓灰质炎病毒的 Enders、Weller 和 Robbins。而 1955 年 Avery 去世后，DNA 方面的诺贝尔奖都比较快，包括 1959 年发错了一半。

注 5：Kossel 获 1910 年诺贝尔化学奖，因为“通过其对蛋白质的研究，包括核物质，对细胞化学知识的贡献”，委员会看重他研究蛋白质，而他的诺贝尔演讲前面大半内容是核酸。Lederberg 于 1946 和 1947 年发表重要结果后，不再念哥伦比亚大学的医学院而转到耶鲁做研究生（Lederberg，1987），他的研究生工作使他在 33 岁与 Beadle 和 Tatum 共享 1958 年诺贝尔奖。Arthur Kornberg（1918-2007）因于 1956 年发现 DNA 多聚酶获 1959 年诺贝尔奖。Perutz 与 Kendrew 因研究血红蛋白和肌球蛋白的三维结构获 1962 年诺贝尔化学奖。Watson 与 Crick 获 1962 年诺贝尔生理或医学奖。Delbrück、Luria 和 Hershey 获 1969 年诺贝尔奖。

注 6：法裔科学家 René Jules Dubos 多才多艺，1948 年因研究土壤细菌及其抗生素与导师 Waksman 一道获得 Lasker 奖（Waksman 因为发现链霉素获 1952 年诺贝尔奖，但他另一研究生 Albert Schatz（1920-2005）认为自己是发现链霉素的主力）。Dubos 被 Avery 招到洛克菲勒以后，除几年在哈佛任教外都在洛克菲勒。他写了很多包括科普的书，其中 1968 年出版的《如此人性的动物》（So Human an

Animal）一书获 1969 年普利策奖。Avery 找到 Dubos 是巧遇，Dubos 于 1927 年研究生毕业时，有人建议他到洛克菲勒求教法国同胞、1912 年诺贝尔奖得主 Alexis Carrel（1873-1944），他们到餐厅午餐时 Dubos 旁边正好坐着 Avery，两人一拍即合，Avery 说了他面临的问题（肺炎球菌荚膜多糖），Dubos 说肯定可以从土壤细菌中找到降解的酶。

注 7：Kossel 于 1884 年发现的组蛋白，在二十世纪末和二十一世纪初为分子生物学研究的热点。1974 年，美国 Stanford 大学的 Roger Kornberg 提出 DNA 重复地、规则地环绕组蛋白形成核小体（Kornberg and Thomas, 1974; Kornberg, 1974）。美国洛杉矶加州大学（UCLA）的 Michael Grunstein 实验室通过酵母遗传学证明组蛋白和核小体对基因转录的重要性。1996 年美国的 David Allis 实验室发现组蛋白乙酰转移酶后（Brownell *et al.*，1996），组蛋白修饰成为很多人研究的表观遗传学的核心问题之一。

注 8：发现肺炎球菌的 Albert Fränkel 因为是犹太人，在 1930 年代已年迈后没有被希特勒得势后的虐犹者所放过，被剥夺教授职位和行医执照。当时看来只是犹太人受迫害，一大批科学家被整和出逃导致有辉煌科学历史的德国迅速落后。

注 9：Dochez 一战时入伍为少校，1919 年到霍普金斯大学任教，1921 年回纽约在哥伦比亚大学任教，长期是 Avery 的室友和朋友。其研究自 1919 年改为链球菌，1928 年改为感冒病毒。

注 10： Horace Judson 的 The Eighth Day of Creation 一般认为是一部很好的有关分子生物学历史的书。但是，他对关键的 Avery 等工作理解不够充分，不清楚 1946 年的文章，且未采访当上还活着的 MacLeod 和 McCarty（McCarty，1985）。

参考文献

Alexander HE and Leidy G (1951) Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. *Journal of Experimental Medicine* 93:345-359.

Alexander HE and Leidy G (1953) Induction of streptomycin resistance in sensitive *Hemophilus influenzae* by extracts containing desoxyribonucleic acid from resistant *Hemophilus influenzae*. *Journal of Experimental Medicine* 97:17-31.

Alexander HE and Redman W (1953) Transformation of type specificity of meningococci: Change in heritable type induce by type-specific extracts containing desoxyribonucleic acid. *Journal of Experimental Medicine* 97:797-806.

Alloway JL (1932) The transformation *in vitro* of R pneumococci into S forms of different specific types by the use of filtered pneumococcus extracts. *Journal of Exerimental Medicine* 55:91-99.

Alloway JL (1933) Further observations on the use of pneumococcus extracts in effecting transformation of type *in vitro*. *Journal of Experimental Medicine* 57:265-278.

Altmann R (1889). Ueber Nucleinsäuren. *Archiv für Anatomie und Physiologie. Physiologische Abteilung*. 524-536, Leipzig.

Amoss HL (1925) The composite nature of a pure culture of a virulent pneumococcus. *Journal of Experimental Medicine* 41:649-662.

Arkwright JA (1921) Variation in bacteria in relation to agglutination both by salts and by specific serum. *Journal of Pathology and Bacteriology* 24:36-60.

Astbury WT (1947) X-ray studies of nucleic acids. Symposium of the Society of Experimental Biology 1:66-76.

Astbury WT and Bell FO (1938) X-ray study of thymonucleic acid. *Nature* 141:747-748.

Austrian R and MacLeod CM (1949) Acquition of M protein by pneumococci through transformation reactions. *Journal of Experimental Medicine* 89:451-460.

Austrian R and Gold J (1964) Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Annals of Internal Medicine* 60:759-776.

Austrian R, Douglas RM, Schiffman G (1976) Prevention of pneumococcal pneumonia by vaccination. *Transactions of the Association of American Physicians* 89:184-189.

Avery OT, Chickering HT, Cole R, Dochez AR (1917) Acute lobar pneumonia: prevention and serum treatment. Monograph No. 7, New York: Rockefeller Institute for Medical Research.

Avery OT and Dubos R (1931) Protective action of a specific enzyme against type III pneumococcus infection in mice. *Journal of Experimental Medicine* 54:73-90.

Avery OT and Goebel WF (1933) Chemoimmunological studies on the soluble specific substance of Pneumococcus: I. the isolation and properties of the acetyl polysaccharide of Pneumococcus type I. *Journal of Experimental Medicine* 58:731-755.

Avery OT, MacLeod CM and McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *Journal of*

*Experimental Medicine* 79: 137-158.
Bernal JD and Crowfoot DM (1934) X-ray photographs of crystalline pepsin. *Nature* 133:794-795.
Beadle GW, Tatum EL (1941). Genetic control of biochemical reactions in *Neurospora*. *Proceedings of the National Academy of Sciences* 27:499-506.
Boivin A (1947) Directed mutation in colon bacilli, by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity. *Cold Spring Harbor Symposium on Quantitative Biology* 12:7-17.
Brachet J (1942) La localization des acides pentosennucléiques dans les tissus animaux et les oeufs d'Amphibiens en voie de développeent. *Archiv de Biologie* 53:207-257.
Brown R (1833) Obesrvation on organs and mode of fecundation in Orchideae and Asclepiadeae. *Transactions of the Linnean Society of London* 16:685-716.
Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, Allis CD (1996). *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* 84:843-851.
Caspersson T (1932) Die quantitative bestimmung vom Thymonucleinsäure mittels fuchsinschwefliger Säure. *Biochem Zeitschtift* 353:97-.
Caspersson T (1935). Ueber die localization der nucleinsäure im Zellkern. *Naturwissenschaften* 30:527-.
Casspersson T (1936). Ueber den chemischen Aufbau der strukturen des Zellkernes. *Skan Arch Physiol* 73(Suppl. 8):1-51.
Caspersson T, Hammarsten E and Hammarsten H (1935). Interactions of proteins and nucleic acid. *Transactions of the Faraday Society* 31:367-389.
Caspersson T and Schultz J (1938) Nucleic acid metabolism of the chromosomes in relation to gene reproduction. *Nature* 142:294-295.
Caspersson T and Schultz J (1939). Pentose nucleotides in the cytoplasm of growing tissues. *Nature* 143:602.
Chargaff E (1950) Chemical specificity of nucleic acids and mechanism of their enzymic degradation. *Experientia* 6:201-209.
Chargaff E and Zamenhof S (1948) The isolation of highly polymerized dexoxypentosenucleic acid from yeast cells. *Journal of Biological Chemistry* 173:327-335.
Chargaff E, Vischer E, Doniger R, Green C and Misani F (1949) The composition of the desoxypentose nucleic acids of thymus and spleen. *Journal of Biological Chemistry* 177:405-416.
Chargaff E, Lipshitz R, Green C and Hodes ME (1951) The composition of the deoxyribonucleic acid of Salmon sperm. *Journal of Biological Chemistry* 192:223-230.
Cobb M (2014) Oswald Avery, DNA, and the transformation of biology. *Current Biology* 24:R55-R60.
Dahm R (2005). Friedrich Miescher and the discovery of DNA. *Developmental Biology* 278:274-288.
Dahm R (2008). Discovering DNA: Friedrich Miescher and the early years of nucleic

acid research. *Human Genetics* 122:565-581.
Dale HH (1946) Address of the president. *Proceedings of the Royal Society B* 133:123-139.
Darnell J (2011) RNA, life's indispensable molecule. pp. 43-44, Cold Spring Harbor Laboratory Press, New York.
Dawson MH (1928) The interconvertibility of "R" and "S" forms of pneumococcus. *Journal of Experimental Medicine* 47:577-591.
Dawson MH (1930) The transformation of pneumococcal types: II the interconvertibility of type-specific S pneumococci. *Journal of Experimental Medicine* 51:123-147.
Dawson MH and Sia RHP (1931) *In vitro* transformation of pneumococcal types. I. A technique for inducing transformation of pneumococcal types *in vitro*. *Journal of Experimental Medicine* 54:681-699.
Dubos RJ (1976) The professor, the institute, and DNA. Rockefeller University Press, NY.
Dobzhansky T (1941) Genetics and the origin of the species, p. 47, Columbia University Press, New York.
Dochez AR and Avery OT (1917a). Soluble substance of pneumococcus origin in the blood and urine during lobar pneumonia. *Proceedings of the Society of Experimental Biology and Medicine* 14:126-127.
Dochez AR and Avery OT (1917b). The elaboration of specific soluble substance by pneumococci during growth. *Journal of Experimental Medicine* 26:477-493.
Dochez AR and Gillespie LJ (1913) A biologic classification of pneumococcus by means of immunity reactions. *Journal of the American Medical Association* 61:727-732.
Dochez AR and Avery OT (1916) Antiblastic immunity. *Journal of Experimental Medicine* 23:61-68.
Downie AW (1972) Pneucococcal transformation-a backward view: Fourth Griffith Memorial Lecture. *Journal of General Microbiology* 73:1-11.
Dunn LC (1951) Genetics in the Twentieth Century: Essays on the Progress of Genetics during its First Fifty Years. Macmillan, NY.
Feulgen R and Rossenbeck H (1924). Mikroskopisch-chemischer Nachweis einer Nucleinsäure von Typus der Thyomonucleinsäure and die darauf beruhende elective Färbung von Zellkernen in microskopicschen Praparaten. *Hoppe-Seyler's Z. Physiol. Chem*. 135: 203-248.
Fränkel A (1884) Über die genuine Pneumonie. Verhandlungen des Congresses für Innere Medicin, Dritter Congress 3:17-31.
Franklin R and Gosling RG (1953) Molecular configuration in sodium thymonucleate. *Nature* 171:740-741.
Goebel WF, Avery OT, Babers FH (1934). Chemo-immunological studies on conjugated carbohydrate proteins. IX. The specificity of antigens prepared by combining the p-aminophenol glysides of disaccharides with protein. *Journal of Experimental Medicine* 60:599-617.
Griffith F (1922) Types of pneumococci obtained from cases of lobar pneumonia. In

*Reports on Public and Medical Subjects*, № 13, pp. 20-45, His Majesty's Stationery Office, London.

Griffith F (1923) The influence of immune serum on the biological properties of pneumococci. In *Reports on Public and Medical Subjects*, № 18, pp. 1-13. His Majesty's Stationery Office, London.

Griffith F (1928) The significance of pneumococcal types. *Journal of Hygiene* 27:113-159.

Heidelberger M and Avery OT (1923) The soluble specific substance of Pneumococcus. *Journal of Experimental Medicine* 38:73-79.

Heidelberger M (1977) A "pure" organic chemist's downward path. *Annual Review of Microbiology* 31:1-12.

Henriques-Normark B and Tuomanen EI (2013) The pneumococcus: epidemiology, microbiology and pathogenesis. *Cold Spring Harbor Perspective* doi:10.1101.

Hershey AD and Chase M (1952) Independent functions of viral protein and nucleic in the growth of bacteriophage. *Journal of General Physiology* 36:39-56.

Hoppe-Seyler EFI (1871) Ueber die chemische Zusammensetzung des Eiters. *Medisch-chemische Untersuchungen* 4:486.

Hotchkiss RD (1948) The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. *Journal of Biological Chemistry* 175:315-332.

Hotchkiss RD (1951) Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. *Cold Spring Harbor Symposium on Quantitative Biology* 16:457-461.

Hotchkiss RD (1952) The biological nature of the bacterial transforming factors. *Experimental Cell Research* Supplement 2:383-389.

Hotchkiss RD (1965) Oswald T Avery: 1877-1955. *Genetics* 51:1-10.

Itakura K, Hirose T, Crea R, Riggs AD, Heyneker HL, Bolivar F, Boyer HW (1977) Expression in *Escherichia* coli of a chemically synthesized gene for the hormone somatostatin. *Science* 198:1056-1963.

Jones W (1914) Nucleic Acids: Their Chemical Properties and Physiological Conduct. Longmans, Green and Co., London.

Jones ME (1953). Albrecht Kossell, a biographical sketch. *Yale Journal of Biology and Medicine* 26:80-97.

Judson H (1979) The eighth day of creation: makers of the revolution in biology. Simon and Schuster.

Kasten FH (2003) Robert Feulgen and his histochemical reaction for DNA. *Biotechnic and Histochemistry* 78:45-49.

Kornberg R and Thomas JO (1974). Chromatin structure: oligomers of the histones. *Science* 184:865-868.

Kornberg R (1974). Chromatin structure: a repeating unit of histones and DNA. *Science* 184:868-871.

Kossel A (1879). Ueber das Nuclein in der Hefe I. *Zschar Physiol Chem* 3:284-291.

Kossel A (1891) Ueber die chemische Zusammensetzung der Zelle. *DuBois-Reymond's Arch* 181:181-186.

Kossel A (1912) The proteins. *Bulletin of the Johns Hopkins Hospital* 23:65-76.
Kunitz M (1940) Crystalline ribonuclease. *Journal of General Physiology* 24:15-32.
Kunitz M (1948) Isolation of crystalline desoxyribonuclease from beef pancreas. *Science* 108:19-20.
Kunitz M (1950) Crystalline desoxyribonuclease. *Journal of General Physiology* 35:349-377.
Lederberg J (1947) Gene recombination and linked segregations in *Escherichia coli*. *Genetics* 32:505-525.
Lederberg J (1952) Cell genetics and hereditary symbiosis. *Physiological Reviews* 32:403-430.
Lederberg J (1956) Genetic transduction. *American Scientist* 44: 264-280.
Lederberg J (1994) The transformation of genetics by DNA: an anniversary celebration of Avery, MacLeod and McCarty (1944). *Genetics* 136:423-426.
Lederberg J (1987) Genetic recombination in bacteria: a discovery account. *Annual Review of Genetics* 21:23-46.
Lederberg J and Tatum EL (1946) Gene recombination in *Escherichia coli*. *Nature* 158:558.
Levene PA, Mandel JA (1908) Über die Darstellung und Analyse einiger der Nucleinsäuren. XIII. Mitteilung. Über ein Verfahren zur Gewinnung der Puribasen. *Biochemische Zeitschrift* 10:215-220.
Levene PA (1909) Über die Hefenucleinsaeure. *Biochemische Zeitschrift* 17:120-121.
Levene PA and Jacobs WA (1912). On the structure of thymus nucleic acid. *Journal of Biological Chemistry*12: 411-420.
Levene PA (1917a). The structure of yeast nucleic acid. *Journal of Biological Chemistry* 31:591-598.
Levene PA (1917b). The chemical individuality of tissue elements and its biological significance. *Journal of American Chemical Society* 39:828-837.
Levene PA (1919). The structure of yeast nucleic acid: IV. ammonia hydrolysis. *Journal of Biological Chemistry* 40: 415-424.
Levene PA (1920a). The structure of yeast nucleic acid: V. ammonia hydrolysis. *Journal of Biological Chemistry* 41:19-23.
Levene PA (1920b). The structure of yeast nucleic acid: ammonia hydrolysis: the so called trinucleotide of Thannhauser and Dorfmüller. *Journal of Biological Chemistry* 43:379-382.
Levene PA and London ES (1929). The structure of thymonucleic acid. *Journal of Biological Chemistry* 83:793-802.
Levene PA and Bass LW (1931) Nucleic Acids. Chemical Catalog Company, New York.
Levene PA Tipson RS (1935) The ring structure of thymidine. *Journal of Biological Chemistry* 109:623-630.
Liu S and Wu H (1938) Mechanism of the recovery of antibody recovery from immune precipitate. *Chinese Journal of Physiology* 13:449-462.
Lubavin (1871) Ueber die künstliche Pepsin-Verdauung des Caseins und die Einwirkung von Wasser auf Eiweissubstanzen. *Medisch-chemische*

*Untersuchungen* 4:485.
Luria SE, Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28:491–511.
MacLeod CM, Hodges RG, Heidelberger M, Bernhard WG (1945) Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *Journal of Experimental Medicine* 82:445-465.
Mandel JA, Levene PA (1905) Darstellung und Analyse einiger der Nucleinsäuren. XI. Mitteilung. Über die Nucleinsäure der Kuhmilchdrüse. *Hoppe-Seyler's Zeitschrift für physiolgische Chemie* 46:155-158.
McCarty M (1945) Reversible inactivation of the substance inducing transformation of pneumococcal types. *Journal of Experimental Medicine* 81:501-514.
McCarty M (1946a) Purification and properties of desoxyribonuclease isolated from beef pancreas. *Journal of General Physiology* 29:123-139.
McCarty M (1946b) Chemical nature and biological specificity of the substance inducing transformation of pneumococcal types. *Bacteriology Reviews* 10:63-71.
McCarty M and Avery OT (1946a) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonucleose on the biological activity of the transforming substance. *Journal of Experimental Medicine* 83:89-96.
McCarty M and Avery OT (1946b) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. III. An improved method for the isolation of the transforming substance and its application to pneumococcus types. *Journal of Experimental Medicine* 83:97-104.
McCarty M (1985) The transforming principle, discovering that genes are made of DNA. Norton, NY.
Miescher JF (1871a) Ueber die chemische Zusammensetzung der Eiterzellen. *Medisch-chemische Untersuchungen* 4:441-460.
Miescher JF (1871b) Die Kerngebilde im Dotter des Hühnereis. *Medisch-chemische Untersuchungen* 4:502.
Miescher JF (1874) Die Spermatozoen einiger Wirbelthiere. Ein Beitrag zur Histochemie. *Verhandlungen der Naturforschenden Gesellschaft in Basel* 6: 138-208.
Mirsky AE (1951). Some chemical aspects of the cell nucleus, in Leslie C. Dunn, ed., *Genetics in the 20th Century*, pp. 127-153, New York.
Mirsky AE, Pollister AW (1942) Nucleoproteins of cell nuclei. *Proceedings of the National Academy of Sciences* 28:344-352.
Mirsky AE, Pollister AW (1946) Chromosin, a desoxyribose nucleoprotein complex of the cell nucleus. *Journal of General Physiology* 30:117-148.
Mirsky AE, Ris H (1947) The chemical composition of isolated chromosomes. *Journal of General Physiology* 31:7-18.
Mirsky AE, Ris H (1949) Variable and constant components of chromosomes. *Nature* 163:666-667.
Mirsky AE and Ris H (1951) The desoxyribonucleic acid content of animal cells and its evolutionary significance. *Journal of General Physiology* 34:451-462.

Morgan TH (1910) Sex-linked inheritance in Drosophila. *Science* 32:120-122.
Morgan AH, Sturtevant AH, Muller HM, and Bridges CB (1915). The mechanisms of Mendelian heredity. Henry Holt & Co., New York.
Neufeld F and Händel L (1909). Über Herstellung und Prüfung von Antipneumokokken-Serum und über die Aussichten einer spezifischen Behandlung der Pneumonie. *Zeitschrift für Immuntätsforschun* 3:159-171.
Neusfld f and Händel L (1912). Zur Frage der Serumtherapie der Pneumonie und der Werbestimmung des Pneumokokken-Serums. *Berliner Klinische Wochenschrift* 49:680-683.
Neufeld F and Levinthal W (1928) Beiträge zur Variabilität der Pneumokokken. *Zeitschtift für Immunitätsforschung* 55:324-340.
Osborne TB, Harris IF (1902) Die Nucleinsäure des Weizenembryos. *Hoppe-Seyler's Zeitschrift für physiolgische Chemie* 36:85-123.
Pasteur L (1880) De l'atténuation du virus du choléra des poules. *C R Acad Sci* 91:673-680.
Pasteur L (1881) Note sur la maladie nouvelle provoquée par la salive d'un enfant mort de la rage. *Bulletin de l'Académie de Médicine (Paris)* 10:94-103.
Piccard J (1874) Über Protamin, Guanin und Sarkin als Bestandteile des Lachssperma. *Chemische Berichte* 7:1714-1719.
Plósz P (1871) Ueber das chemische Verhalten der Kerne de Vogel- und Schlangenblutkorperchen. *Medisch-chemische Untersuchungen* 4:463-484.
Reichard P (2002) Oswald T Avery and the Nobel Prize in medicine. *Journal of Biological Chemistry* 277:13355-13362.
Reimann HA (1925) Variations in specificity and virulence of *Pneumococci* during growth *in vitro*. *Journal of Experimental Medicine* 41:587-600.
Reimann HA (1927) The occurrence of degraded pneumococci in vivo. *Journal of Experimental Medicine* 45:807-814.
Reimann HA (1929) The reversion of R to S *Pneumococci*. *Journal of Experimental Medicine* 49:237-249.
Schultz J and Caspersson T (1940) Ribonucleic acids in both nucleus and cytoplasm and the function of the nucleolus. *Proc Natl Acad Sci USA* 26:507-515.
Schultz J (1941) The evidence of the nucleoprotein nature of the gene. *Cold Spring Harbor Symposium on Quantitative Biology* 9:151-167.
Schultz J (1943) Physiological aspects of genetics. *Annual Review of Physiology* 5:35-62.
Sevag MG (1934) Eine neue physikalische enteiweissungemethode zur darstellung biologisch wirksamer subsanzen. *Biochem Z* 273:419.
Sia RHP (1926) Presence of type specific pneumocco-opsonins in sera of animals naturally resistant to pneumococcus infection. *Experimental Biology and Medicine* 24:709-711.
Sia RHP and Dawson MH (1931) *In vitro* transformation of pneumococcal types II. The nature of the factor responsible for the transformation of pneumococcal types. *Journal of Experimental Medicine* 54: 701-710.
Signer R, Caspersson T, Hammarsten E (1938) Molecular shape and size of the

thymonucleic acid. *Nature* 141:122.
Sternberg GM (1881) A fatal form of septicaemia in the rabbit produced by the subcutaneous injection of human saliva. An experimental research. *Bulletin of the National Board of Health* 2:781-783.
Stryker LM (1916) Variations in the pneumococcus induced by growth in immune system. *Journal of Experimental Medicine* 24:49-68.
Steudel H (1906) Die Zusammensetzung der Nucleinsäuren aus Thymus und das Heringsmilch. *Hoppe-Seyler's Zeitschrift für physiolgische Chemie* 49:406-409.
Tamm C, Shapiro HE, Lipshitz R and Chargaff E (1953) Distribution density of nucleotides within a desoxyribonucleic acid chain. *Journal of Biological Chemistry* 203:673-688.
Thomas R (1992) Molecular genetics under an embryologist's microscope: Jean Brachet, 1909-1988. *Genetics* 131:515-518.
Thompson RHS and Dubos RJ (1938) The isolation of nucleic acid and nucleoprotein fractions from pneumococci. *Journal of Biological Chemistry* 125:65-74.
Vischer E and Chargaff E (1947) The separation and characterization of purines in minute amounts of nucleic acid hydrolysates. *Journal of Biological Chemistry* 168:781-782.
Vischer E and Chargaff E (1948) The separation and quantitative estimation of purines and pyridines in minute amounts. *Journal of Biological Chemistry* 176:703-714.
Vischer E, Zamenhof S and Chargaff E (1949) Microbial nucleic acids: the desoxypentose nucleic acids of avian tubercle bacilli and yeast. *Journal of Biological Chemistry* 177:429-438.
Watson JD (1968) Double helix. Atheneum, New York.
Watson JD and Crick FHC (1953a) A structure for deoxyribose nucleic acid. *Nature* 171:737-738.
Watson JD and Crick FHC (1953b) Genetical implications of the structure of deoxyribonucleic acid. *Nature* 171:964-967.
Wilkins MHF, Stokes AR and Wilson HR (1953) Molecular structure of deoxypentose nucleic acids. *Nature* 171:738-740.
Wilson EB (1925) The cell in development and inheritance. Macmillan.
Wright AE, Morgan WP, Colebrook L, Dodge RW (1914). Observations on prophylactic inoculation against pneumococcus infections and on the result which have been achieved by it. *Lancet* 1:1-10, 87-95.
Zamenhof S and Chargaff E (1950) Dissymmetry in nucleotide sequence of desoxypentose nucleic acids. *Journal of Biological Chemistry* 187:1-14.

本章要求阅读文献：

Griffith F (1928) The significance of pneumococcal types. *Journal of Hygiene* 27:113-159.

Avery OT, MacLeod CM and McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *Journal of Experimental Medicine* 79: 137-158.

VOLUME XXVII JANUARY, 1928 No. 2

# THE SIGNIFICANCE OF PNEUMOCOCCAL TYPES.

BY FRED. GRIFFITH, M.B.

(*A Medical Officer of the Ministry of Health.*)

(*From the Ministry's Pathological Laboratory.*)

CONTENTS.



## I. OBSERVATIONS ON CLINICAL MATERIAL.

SINCE communicating my report[1] on the distribution of pneumococcal types in a series of 150 cases of lobar pneumonia occurring in the period from April, 1920 to January, 1922, I have not made any special investigation of this subject. In the course, however, of other inquiries and of the routine examination of sputum during the period from the end of January, 1922, to March, 1927, some further data have been accumulated[2].

Table I gives the results in two series and, for comparison, those previously published.

[1] *Reports on Public Health and Medical Subjects* (1922), No. 13.

[2] I owe many thanks to Dr J. Bell Ferguson, formerly Medical Officer of Health for Smethwick, for sending me many specimens from cases of lobar pneumonia.

Table I. *Types of Pneumococci in Lobar Pneumonia.*

| Period of inquiry | Total cases examined | Percentage incidence of types | | | |
|---|---|---|---|---|---|
| | | Type I | Type II | Type III | Group IV |
| Apr. 1920–Jan. 1922 | 150 | 30·6 | 32·6 | 6·6 | 30·0 |
| Feb. 1922–Oct. 1924 | 61 | 42·6 | 21·3 | 3·2 | 32·7 |
| Nov. 1924–Mar. 1927 | 67 | 34·3 | 7·4 | 4·4 | 53·7 |

The main point of interest, since the beginning of the inquiry, is the progressive diminution in the number of cases of pneumonia attributable to Type II pneumococcus. The great majority of the cases occurred in the Smethwick district, and the figures may reveal a real local decrease of Type II, and a corresponding increase of Group IV cases. It must, however, be remembered that the isolation on a single occasion of a Group IV strain from a sputum, especially in the later stages of the pneumonia, does not prove that strain to be the cause of the disease. This is clearly shown by the examination of several samples of sputum taken at different times from the same case; in these a Group IV strain was often found in addition to one or other of the chief types. There may be a slight element of uncertainty regarding causal connection of the Group IV strains with the pneumonia, since the cultures of pneumococci in this series were derived from sputum (except in four cases where the material was pneumonic lung) and some of the samples of sputum were obtained when the disease had been in progress for some time—from 4 to 11 days after the onset.

## Occurrence of a Variety of Serological Types in the Sputum from an individual case of pneumonia.

In my report (1922 *loc. cit.*) I described a number of instances where several serological varieties of pneumococci were found in the sputum of a pneumonia patient. One instance was particularly striking, where the sputum, No. 112, taken on the sixth day of the disease (a crisis had not occurred), yielded a Type I culture and three strains of Group IV, *i.e.* four distinct serological races. On other occasions different specimens of sputum from the same case, taken at varying periods after the onset of pneumonia, were found to contain two or more serological types.

Three alternative explanations, at least, are possible.

1. The patient having previously been a carrier of several Group IV strains became infected with a Type I strain which produced pneumonia. There is no evidence to show which of the types was present in the pneumonic lung, but I think that the Type I may be assumed to have caused the disease.

2. The patient when normal was a naso-pharyngeal carrier of a Group IV strain. Owing to a condition being produced favourable to mutation, a type of pneumococcus, in this instance Type I, was evolved in his air-passages which was able to set up pneumonia. On this hypothesis, the different serological types would be evidence of the progressive evolution.

3. On the other hand, the Group IV strains might be derived from the Type I in the course of successful resistance against the latter strain. With the increase of immune

substances or tissue resistance the Type I would be finally eliminated, and there would remain only the Group IV strains which are almost certainly of lower infectivity and perhaps of less complex antigenic structure.

In the hope of gaining further information on these points I continued the analysis of the types yielded by the same patient, employing the following method:

The sputum was preserved in the ice-chest until the preliminary diagnosis of the infecting pneumococcus had been made in the usual way, viz. by the intraperitoneal inoculation of a mouse and by testing the peritoneal washings versus the type sera. The following day some of the sputum was inoculated together with the type serum corresponding to the strain identified. Frequently the second mouse died from an infection with a pneumococcus of a different type from that first obtained. If a serum corresponding to the fresh type was available a third mouse was inoculated together with the sera appropriate to the two types already identified. The following examples will make the procedure clear.

(1) A specimen of sputum, No. 239, from a case of pneumonia of four days' duration was sown on plates, and five colonies of pneumococci were examined, all of which proved to be Type I; the mouse test also gave Type I. The next day the sputum was inoculated into a mouse together with Type I serum. The mouse died and the peritoneal washing reacted only with Pn. 41 (Group IV) serum; the blood of the mouse was plated and of five colonies examined two were Pn. 41 and three were strains of Group IV which could not be identified. The sputum was inoculated a third time plus a mixture of Type I and Pn. 41 sera. The third mouse died within 24 hours and its blood yielded a virulent culture of a Group IV strain which did not react with any of the available agglutinating sera.

(2) A more complete examination was made of the sputum from a second case, No. 273, of lobar pneumonia, specimens being taken at different periods after the onset of the disease. The details are given in Table II.

Table II.

| | | Types of pneumococci obtained | | |
|---|---|---|---|---|
| | | | Through mouse | |
| Specimen of sputum | Day of disease | On direct plate | Sputum alone | +Type I serum |
| 1 | 4th | Type I (3 colonies) | Type I | Pn. 41 |
| 2 | 6th | Type I (7 colonies) | Type I | Pn. 160 and Group IV? |
| 3 | 8th | Not done | Type I and Pn. 160 | — |
| 4 | 12th | Not done | Type I | — |
| 5 | 15th | Group IV (1 colony) | Group IV | — |
| 6 | 17th | Pn. 160 (2 colonies) | Group IV | — |
| 7 | 19th | Not done | Type I and Pn. 160 | — |
| 8 | 21st | No Pn. colonies | Type I | — |

The first specimen of sputum collected on the 4th day of the disease yielded pneumococci of Type I, both on the direct plate and through the mouse. The same specimen of sputum, which had been kept on ice in the meantime, was inoculated into a mouse together with a protective dose of Type I serum; the mouse died within 24 hours and a pure culture of Group IV, viz. Pn. 41, was obtained from the blood. The specimen collected on the 6th day of the disease was examined in the same way and gave a similar result, but on

this occasion the protected mouse yielded two different strains of Group IV. One of the latter was identified with Pn. 160; I had no serum corresponding to the other.

An interesting result was obtained with the 8th day specimen. The mouse inoculated with the sputum died of a mixed infection, and on a plate from the blood it was possible to pick out only three pneumococcus colonies. Two of these colonies reacted with Pn. 160 serum alone, while the third gave equally good specific clumping (firm masses) with both Type I and Pn. 160 sera. This third colony culture was plated and the plate showed two varieties of smooth pneumococcus colonies differing slightly in appearance but easily distinguishable. Several of each variety were grown in broth and the agglutination reactions were tested; rounded dome-shaped pearly colonies were found to be Type I, and larger, flatter and more translucent colonies were Pn. 160. In addition to the above there were on the plate a few typical rough pneumococcus colonies, four of which were subcultivated and tested on mice. Three were avirulent but the fourth caused septicaemia in mice and produced a peritoneal washing which agglutinated specifically with Pn. 160 serum. The culture obtained from the blood remained rough in character and thus possessed a combination of rough cultural characteristics and virulence which had not previously been noted. Further experiments were made with this culture (see p. 117).

The above instance may be simply an example of a mixed colony and nothing more. On the other hand there is the possibility that this mixed colony was derived from a single coccus possessing double antigenic properties. The culture being perhaps in an unstable condition may have separated in the course of growth into two elements, Type I and Pn. 160, in each of which the second antigen was suppressed.

On the 12th day the sputum yielded Type I through the mouse. In a specimen taken three days later, Type I was not found, the direct plate cultures as well as the cultures through two mice belonging to Group IV but not reacting with any of the available sera.

On the 17th day of the disease there was again no evidence of Type I, each of two mice being infected with an unknown Group IV strain, while on the direct plate Pn. 160 reappeared. Type I was again found on the 19th day and still persisted in the sputum on the 21st day after the onset of the pneumonia.

It is curious that the Pn. 41 culture was never again found after the first test. The strain produced large capsules in the blood of the mouse and a peritoneal washing reacted vigorously with the type serum. The Pn. 160 culture on the other hand appeared frequently in the course of the investigation.

(3) Sputum 218 came from a man who had been ill with pneumonia and had not yet had a crisis. This specimen yielded Type I colonies both on plates made direct from the sputum and through the mouse. On the 10th day of the disease, three days after the crisis, a second specimen of sputum was taken. The sputum alone inoculated into a mouse caused fatal septicaemia, and plates from the blood gave a pure growth of pneumococci; three colonies belonged to Type I and seven to an unidentified strain of Group IV. Inoculated plus Type I serum this 10th day sputum killed a mouse and ten plate colonies from the blood belonged to an unidentified Group IV strain.

On the 14th day of the pneumonia the sputum alone killed a mouse, and four colonies on the plate from the blood were identified as Type I. Sputum together with Type I serum yielded through the mouse an apparently pure culture of Type II B (12 plate colonies were identified). The sputum which had been preserved in the ice-chest was then inoculated into a mouse together with a mixture of Types I and II B sera; this mouse died of a pure Type III infection.

A final specimen of sputum taken on the 16th day of disease was inoculated into three mice; (1) with sputum alone and (2) sputum plus Type I serum both yielded Type II B only, (3) sputum plus a mixture of Types I and II B sera yielded an unidentified strain of Group IV.

(4) Six other cases of pneumonia were investigated in a similar manner to the above.

Type I pneumococci were grown from each case and, in addition, Group IV strains were obtained from five and a Type III strain from one.

(5) A few cases only of lobar pneumonia due to Type II pneumococci have been studied in the above manner. Sputum No. 267, which killed a mouse with a Type II infection, was re-inoculated plus Type II serum and the mouse died of a Type III infection.

(6) The lung from a fatal case of pneumonia, No. 230, was plated directly and 34 colonies were examined, all of which proved to be Type II; 12 colonies from a mouse inoculated with the lung were also Type II.

I have not had an opportunity of ascertaining whether more than one type of pneumococcus can be obtained from the lung in a fatal case of pneumonia due to Type I; only in sputum has a mixture of several types been demonstrated. This latter fact might suggest that the secondary strains, viz. Group IV and Type III, were present in the upper air passages prior to the infection with the more invasive strains of Types I and II. On a balance of probabilities interchangeability of type seems a no more unlikely hypothesis than multiple infection with four or five different and unalterable serological varieties of pneumococci. Moreover, failure to find more than one type in the lung of a fatal case of pneumonia would not be conclusive evidence against the modification hypothesis, since the fatal termination would in itself indicate an absence of those protective antibodies which may be necessary to initiate an alteration in the type of the infecting pneumococcus. Lung puncture in a case of resolving pneumonia might furnish more precise indications.

The above findings, taken alone, are not decisive in favour of either of the two hypotheses, but they assume greater significance when considered together with the laboratory experiments on alteration of type described later.

## Further remarks on a Pneumococcus Strain from Sputum producing rough colonies yet virulent for mice.

A distinguishing feature of an avirulent pneumococcus is the rough appearance of the colonies after 24 hours' growth on a blood agar plate. Until the appearance of the strain already referred to on p. 116 the above morphological character of a pneumococcus colony has been found invariably associated with absence of virulence. The strain in question produced very typical rough colonies, but nevertheless was able to multiply in the mouse and cause fatal septicaemia. The blood of the mouse showed pneumococci with well marked capsules, and on plate cultures rough colonies grew, identical in appearance, except in one instance, with those of the original strain.

There are some points of interest both in regard to the origin of the strain and in the experiments which proved it to combine roughness of colony with virulence for mice.

The strain was derived from the sputum, No. 273, of a case of lobar pneumonia. Several specimens of sputum from this case were examined at different stages of the disease and the results are given on p. 116. The sputum which yielded this strain was the third specimen and was taken on the 8th day after the onset of pneumonia; it produced pneumococcal

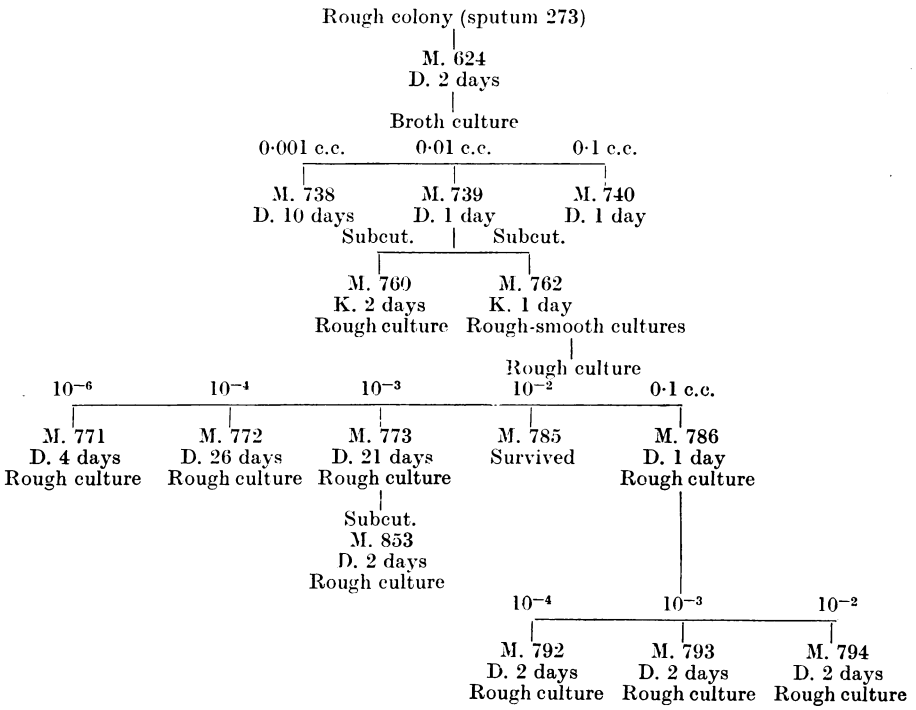
septicaemia in a mouse and a smooth colony was subcultivated in broth from the blood. The broth culture on being tested against all the available pneumococcus agglutinating sera gave a positive reaction with two sera, viz. Type I and Group IV (Pn. 160); in both cases the coarse masses characteristic of a reaction with soluble substance were formed. This unusual occurrence was investigated in the following ways.

The original colony culture in broth was plated and on the plate three different varieties of colonies were identified. Two varieties were smooth, one of which was found to agglutinate with Type I serum and the other with Group IV (Pn. 160) serum; the third variety was rough. Four of the rough colonies were subcultivated and each was inoculated subcutaneously into a mouse in a dose of 0·25 c.c. of broth culture. Three of the mice were well when killed three days later and cultures were grown from the seat of inoculation; each culture thus obtained was inoculated intraperitoneally into a second mouse without causing any ill effects. The mouse inoculated with the fourth colony died of pneumococcal septicaemia. The blood yielded colonies which were wholly of the rough variety and which, nevertheless, when grown in broth agglutinated typically with Pn. 160 serum.

The broth culture of the original colony was also inoculated intraperitoneally in a dose of 0·01 c.c. into three mice which had received preliminary treatment with protective sera. The mouse immunised with Type I serum died 6 days after inoculation and a pure culture of Pn. 160 was obtained from the blood; the mouse protected with Pn. 160 serum died within 24 hours of a Type I infection; the third mouse treated with the two sera, Type I and Pn. 160, survived the culture inoculation. The colonies of the two strains obtained as above were smooth; the rough Pn. 160 element was eliminated.

A series of passage experiments, the details of which are given in Table III, was made with the rough virulent culture of Pn. 160 to ascertain whether a change into the smooth form might be induced.

Table III.



Throughout a long series of passage experiments, of which those given in Table III are about half the total, the majority of the mice died of pneumococcal septicaemia and showed capsulated diplococci in the blood; the cultures obtained from the blood retained the original rough appearance of colonies with a single exception. The survival of some of the mice for prolonged periods, up to 26 days, is noteworthy; in the end such mice succumbed to pneumococcal septicaemia and yielded rough colonies from the blood. All the rough cultures in broth agglutinated with Pn. 160 serum.

At one stage of the passage experiment, as mentioned above, a change from rough to smooth occurred and this was the only instance observed, although plate cultures had been made from every mouse in the series. The change from the rather large coherent colony into a much smaller shiny colony of almost watery consistency was very striking.

The circumstances of the conversion have some interest. The mouse, No. 762, from which the smooth variety was obtained, had been inoculated subcutaneously with 0·25 c.c. of broth culture of a rough passage strain and was killed the next day. The blood of the mouse yielded numerous colonies of the usual rough character, among which was detected one with a small smooth segment. The latter was touched with a fine needle and from it a second plate culture was made on which grew a mixture of rough and smooth colonies. From one of the latter a pure smooth colony culture was obtained and this agglutinated like the original rough colony with Pn. 160 serum.

The virulence of the smooth colony resembled that of the rough colony and after passage through four mice in series the same chronic infection occurred ending in death from septicaemia; for example, one mouse which received $10^{-5}$ dose of the smooth broth culture died 25 days after inoculation. At the end of the passage the smooth colony still retained its small size, being definitely smaller than the normal Group IV colony.

Both the above cultures, the rough and the smooth, were grown in the antiserum of their type. From the serum cultures rough and smooth strains were obtained and it was found that each variety had become attenuated. Neither would kill mice in intraperitoneal doses ranging from 0·1 c.c. to 0·2 c.c., the mice being kept under observation for two months.

I record the foregoing observations without attempting at present to interpret them.

## A Strain agglutinating specifically with Sera of two Different Types.

The strains of Group IV comprise many different types which are remarkably well defined and exhibit no cross-agglutination amongst themselves, provided one takes as the criterion of type agglutination the formation of firm clumps, either with cultures or with peritoneal exudates. This specificity is no doubt due to the secretion of soluble substances peculiar to each type. The strain which I am about to describe gives with two different type sera the firm clumps characteristic of the reaction between soluble substance and agglutinin.

The source of the culture was the lung of a woman who died 9 days after the onset of broncho-pneumonia. A plate made directly from the lung showed large smooth pneumococcus colonies. Seven colonies were subcultured separately and were inoculated intraperitoneally into mice, which died within 24 hours. The peritoneal washings from these mice were found to react with two different Group IV sera, viz. II B and Pn. 87; the reactions were equal with six of the washings, while the seventh gave a heavier precipitate with II B serum than with Pn. 87 serum.

Comparative tests were made with the new strain and with the stock II B and Pn. 87 strains.

| Strain | | II B serum | Pn. 87 serum |
|---|---|---|---|
| II B: | peritoneal washing | + | − |
| Pn. 87: | ,, | − | + |
| New strain: | ,, | + | + |

The culture of II B agglutinated up to 1 in 320 with II B serum and not at all with Pn. 87 serum. Pn. 87 culture agglutinated to 1 in 160 with Pn. 87 serum and gave a trace of 1 in 20 with II B serum. The new strain in culture agglutinated with both sera but, unlike the two homologous strains, did not form firm masses characteristic of virulent pneumococci. Instead there was produced a turbidity made up of fine granules, showing that there was probably a deficiency of soluble substance. In the peritoneal cavity of the mouse, on the other hand, soluble substance is more readily produced and in consequence the washings gave with both sera the typical reaction of a virulent strain.

It may be remarked that such behaviour has been observed on a few other occasions; a pneumococcus strain in culture, obtained direct from sputum, has not reacted with the type serum while the same strain in the peritoneal exudate of a mouse has reacted typically. Such a result is no doubt an indication of reduced virulence with associated deficiency in the production of soluble substance.

A series of experiments were made to prove that the new strain contained two antigens and was not merely a mixture of II B and Pn. 87. A preliminary test showed that II B and Pn. 87 sera each protected mice against 0·0001 c.c. of the new strain but not against 0·001 c.c. Plate cultures were made from the blood of (1) a mouse injected with II B serum, and (2) a mouse injected with Pn. 87 serum along with the new strain, and four colonies from each were studied. Seven of the colony cultures reacted equally well with both sera; one gave a stronger reaction with II B serum than with Pn. 87 serum. Cultures were made in II B serum and were inoculated into mice treated with II B serum; from the mice which died strains with the double antigens were recovered, whereas if the new strain had been a simple mixture one would have expected the II B constituent to be eliminated. In point of fact the II B antigen was probably the major antigen, since some single colony strains were obtained which reacted only slightly with Pn. 87 serum.

I have recorded my observations on this strain rather fully since it is the only exception I have found to the rule that a pneumococcus has only one well-developed antigen. The observation may however be significant as indicating that this rule is not absolute and that the purity of the specific antigen in virulent pneumococci may only be apparent.

## II. Experimental Modification.

## Production of Attenuated Strains of Pneumococci.

### (1) *By growth in immune serum.*

Culture in homologous immune serum is perhaps the most convenient method of producing attenuated strains of pneumococci, recognisable by the morphological appearances of the colonies and termed the R form of the pneumococcus. Complete attenuation of a virulent pneumococcus culture is secured only after several passages in series, the first and second serum cultures generally being composed of a mixture of S and R forms while either the third or fourth cultures may contain purely R forms. During my first

observations[1] on this matter I found that the more stable R forms were obtained from the later cultures in series and I concluded that this was the effect of the several passages in serum.

As, however, very stable R forms may be isolated from a first serum culture, and, on the other hand, unstable R strains, those which readily regain virulence in the mouse, may retain this property after repeated passages in immune serum, my first experience may have been a matter of chance. It appears, as the tests on p. 123 show, that R colonies on a plate from an immune serum culture are not equally attenuated, the capacity to revert to the smooth type on inoculation into the mouse being more pronounced in some colonies than in others. Thus it is true that several passages in series are required to eliminate the smooth form but it cannot be predicted what the effect of repeated exposure to the action of immune serum may be once the R state has been reached.

(2) *By growth on solid media.*

Attenuated R strains can also be obtained from virulent cultures by growth on chocolate blood agar. A virulent pneumococcus culture in blood broth, plated on this medium and examined after 24 hours' incubation at 37° C., yields as a rule only smooth colonies. If the plate is left in the incubator for two days, some of the smooth discs, which after the first night's incubation were perfectly regular in outline, develop small rough patches in their margins. The rough patches may develop into a wedge with the base at the periphery and the apex at the centre of the colony. They may be either raised above or depressed below the level of the original smooth growth, and generally they project beyond its margin. Sometimes the rough area forms a rounded projection extending well beyond the margin of the S colony and sending a process like a single root towards the centre. Usually a colony shows only a single rough focus but cultures are variable in this respect, and some produce colonies which become studded with rough areas. But many cultures, especially highly virulent strains of Type II, fail entirely to produce colonies with rough patches.

Rough foci have never, in my experience, become visible in smooth colonies after a single night's incubation; it is essential that the culture medium should be sufficiently favourable to allow growth to continue for at least 48 hours. The following is an instance. The stock virulent strains of Types I and II were plated and produced completely smooth colonies after 48 hours' incubation. After three days incubation Type I colonies showed occasional small rough areas but none was seen in the Type II colonies even on the 4th day. One of the Type II colonies was subcultured in blood broth and then plated; the majority of the colonies produced were smooth but there were also a few R colonies.

Apparently a few R pneumococci are formed in a culture which is allowed to age on blood agar and these may multiply and produce a rough area or colony in and perhaps at the expense of the smooth growth.

When the patch is large it may be touched with the point of a spatula and a pure

[1] *Reports on Public Health and Medical Subjects* (1923), No. 18.

rough strain may be obtained. Generally, however, when a rough patch is subcultured in blood broth and plated a mixture of R and S colonies grows.

R cultures from rough areas are, so far as I have ascertained, identical with those obtained by growth in immune serum.

The tendency to produce colonies with rough patches seems to be inherent in some strains and may perhaps indicate deterioration in virulence. It is not removed by a single animal passage; a Type I strain of medium virulence which produced colonies with many rough patches was inoculated into a mouse and caused fatal septicaemia. A culture from the blood was plated and produced a pure growth of smooth colonies after 24 hours' incubation; many of the latter developed rough patches after a second night's incubation.

Rough patches in colonies have been produced only on the chocolate blood medium which contains fresh horse serum and every batch of this medium has not been equally favourable. It is possible that the rough change may be due to the presence of immune bodies in the horse serum. On agar plates without blood pneumococcus colonies quickly lyse and become almost invisible. If such plates are left in the incubator daughter colonies may grow out from the lysed colonies, but when these are subcultivated and plated they almost invariably produce smooth colonies only.

The formation of individual R pneumococci in a smooth culture does not apparently take place when the culture has ceased to grow. This was shown in an experiment made to test the viability of pneumococci on ordinary agar.

Fourteen cultures of pneumococci, each of a different serological type, on nutrient agar slopes were incubated at 37° C. in tubes sealed with paraffined plugs. In 24 hours the growth had become almost invisible from lysis. The tubes were left undisturbed in the incubator for two months when they were scraped and subcultivated in blood broth. All the cultures were viable and the colonies grown on blood agar plates were smooth. After $5\frac{1}{2}$ months' incubation twelve of the cultures were still alive, and on plates, while most of the colonies produced were smooth; occasional rough ones were detected. After 15 months in the incubator four cultures still survived; two were completely rough and had lost their virulence for mice, while the other two produced a mixture of R and S colonies. It will be observed that the surviving pneumococci remained in their original smooth condition in the tubes which had not been disturbed for two months. At the end of that period I suggest that the scraping of the surface and the transference of pneumococci to fresh parts of the medium caused further growth, with the result that R forms appeared. For the same reason the change to the R state was still more advanced at the conclusion of the experiment.

Similarly, as the pneumococci do not grow, no attenuation occurs when the spleens of mice which have died of pneumococcal septicaemia are dried and preserved for prolonged periods. I have recovered strains from dried spleens after $3\frac{1}{2}$ years and have found the virulence unaltered. The surviving pneumococci may be very few in number and may be recovered in the following way. The whole of the spleen is ground to a fine powder in a mortar and emulsified in a small quantity of blood broth. This may be plated directly or after a few hours' incubation. The colonies produced have always been of the smooth form; the pneumococci have remained dormant and there has, therefore, been no opportunity for the production of the R forms.

### (3) *Differences between individual R and S colonies.*

Virulent pneumococci which have been grown in homologous immune serum and have undergone the change from the S to the R form are not all equally affected. Pure R colony cultures show differences amongst themselves in (1) capacity to revert, (2) type of agglutination and (3) immunising properties.

A virulent Type I strain was grown in Type I serum for two generations, the second of which was plated. Six R colonies were taken and grown in small quantities of blood broth; these colonies were identical in appearance and gave non-specific agglutination in pneumococcal type sera. Each colony culture was subcultivated in 10 c.c. of broth; this was centrifuged and the deposits were inoculated into mice subcutaneously.

*1st inoculation experiments.*

| No. of colony culture | Mouse | Result |
|---|---|---|
| 1 | 923 | Died 2 days. S colonies from blood |
| 2 | 924 | ,, ,, ,, |
| 3 | 925 | ,, ,, ,, |
| 4 | 926 | Survived 12 days. Nil in blood |
| 5 | 927 | Died 4 days. S colonies from blood |
| 6 | 928 | Survived |

Each of the six colony cultures was then plated and an isolated colony was grown which was again inoculated subcutaneously in a dose of 10 c.c. of broth culture deposit.

*2nd inoculation experiments.*

| No. of colony culture | Mouse | Result |
|---|---|---|
| 1 | 967 | Died 2 days. S colonies from blood |
| 2 | 968 | ,, ,, ,, |
| 3 | 969 | ,, ,, ,, |
| 4 | 970 | Survived |
| 5 | 971 | Died 3 days. S colonies from blood |
| 6 | 972 | Survived |

In order to make certain that the colony cultures were free from any S forms of pneumococci, each culture was again plated and an isolated R colony was grown in blood broth. This procedure (plating and selection of colonies) was carried out six times in succession. Broth cultures were made from colonies on the final plates and these were inoculated subcutaneously into mice in the same doses as before.

*3rd inoculation experiments.*

| No. of colony culture | Mouse | Result |
|---|---|---|
| 1 | 20 | Died 2 days. Pneumococcal septicaemia, culture overgrown |
| 2 | 21 | Died 2 days. S colonies from blood |
| 3 | 22 | Died 2¼ days. ,, ,, |
| 4 | 23 | Survived |
| 5 | 24 | Died 2 days. S colonies from blood |
| 6 | 25 | Survived |

The results were practically identical in each of the three series of inoculation experiments; four of the R strains reverted in the mouse to the smooth type, while two, Nos. 4 and 6, were more completely attenuated. The different degrees of virulence were retained after seven successive platings, thus showing that the characters were stable and were the property of the whole strain in each case.

The following experiment reveals similar differences between individual R colonies.

Four cultures made from different colonies of a rough Type I strain were inoculated into mice in doses of 0·15 c.c. intraperitoneally. Cultures 1 and 2 killed the mice in two days and S colonies were grown from the blood. The mice inoculated with cultures 3 and 4 survived. The latter two mice were reinoculated on the 6th day with 0·25 c.c. and on the 13th day with 0·5 c.c., each with the same living broth culture as before. They were tested on the 26th day after the first inoculation with $10^{-6}$ c.c. of a virulent Type I culture. Both mice died, thus showing absence of immunity.

The immunising capacity of the other two rough cultures, 1 and 2, was tested in the following way.

No. 1 was inoculated intravenously into three mice, each with 0·2 c.c. of living broth culture. They resisted this inoculation and a test dose of virulent culture was given 18 days later. All of these survived. A similar intravenous experiment with No. 2 failed to produce immunity against the same test dose. No. 2 was then used to vaccinate 3 mice, inoculated intraperitoneally twice at 7 days' interval. Tested 21 days after the first vaccinating dose, two out of these mice survived.

Thus two of the rough cultures produced immunity and two failed. The properties of the four strains are given in the following table:

| No. of rough culture | Reversible to S type | Immunising capacity | Agglutinability |
|---|---|---|---|
| 1 | Yes | Positive | Type specific |
| 2 | ,, | ,, | ,, |
| 3 | No | Negative | Non-specific |
| 4 | ,, | ,, | ,, |

In this instance by type specific in regard to agglutinability is meant that though agglutination was of the rough character, *i.e.* the deposit was readily shaken up, it occurred up to the full titre 1 in 160 of a smooth Type I serum. The non-specific cultures agglutinated to 1 in 10 only with both Types I and II sera.

An experiment on similar lines was made with a rough Type II culture.

Six rough strains from individual colonies were inoculated into mice in doses of 0·75 c.c. and 0·1 c.c. of broth culture intraperitoneally. All the mice which received the larger dose died within 24 hours, but in no case was there any reversion to the smooth type. The peritoneal washings were tested against Type II serum and several gave a slight precipitate showing probably the formation of a small amount of soluble substance; two gave no precipitate. The mice injected with 0·1 c.c. all remained well; they were reinoculated after 6 days with 0·2 c.c. and after a further 7 days with 0·3 c.c. of the same cultures as before. The immunity of five mice (one died accidentally) was tested 26 days after the first injection with $10^{-6}$ c.c. of a virulent Type II culture. Three mice resisted and two succumbed.

It is interesting that the two mice which died were immunised with the two cultures which failed to produce a trace of soluble substance in the peritoneal washings. The change to the R form is apparently less complete in some pneumococci than in others, and it is possible that the retention of a small amount of the original S antigen in their composition may explain the capability of certain R strains to immunise against a virulent pneumococcus as well as their tendency to revert to the S form.

On the other hand, while diminution of virulence in a pneumococcus culture may be due to a proportion of the individual organisms composing it having undergone the change into the R form, this is not invariably the case. Cultures which produce only smooth colonies may possess intermediate grades of virulence, killing mice in doses not less than 0·1 c.c. or 0·01 c.c. of broth culture. This has been observed in cultures immediately after reversion from the rough to the smooth form. In such instances the change from the

one form of antigen to the other may not have been complete, and thus larger numbers of organisms are required to produce a sufficient concentration of those substances which neutralise the protective fluids of the animal and enable the bacteria to multiply.

## Reversion from Rough to Smooth.

### A. *Origin of the rough strains used; effect of different sera.*

The rough Type II strain was obtained in the following way. A virulent culture of Type II, which killed mice when inoculated intraperitoneally in a dose of $10^{-8}$ c.c., was sown into undiluted Type II serum and was passed from serum to serum for six generations, each of which was incubated at 37° C. overnight. The final culture was plated, and five rough colonies were selected and subcultured.

A test was made to show that these five subcultures were free from smooth virulent pneumococci; the procedure being the same for each, a single description is applicable to all. Each rough culture was plated and an isolated colony was grown in blood broth. A mouse was inoculated intraperitoneally from the blood broth culture and a plate was made. This procedure, plating followed by selection of rough colony and mouse test, was repeated six times in succession. In no case did any of the plates show a smooth colony, and all the mice (a total of 30) survived the intraperitoneal inoculation of culture, the doses of which ranged from 0·1 c.c. to 1·0 c.c. The final cultures were tested against agglutinating sera of Types I and II with both of which only minute clumps were formed, thus showing that the type characteristics had been lost.

A virulent culture of Type I was treated as above, except that the transferences were limited to five. The final rough cultures were tested on mice in the same way as those of the Type II. Although the appearance of the colonies was typically rough, they were found to revert readily in the mouse to the smooth virulent variety. One culture was then passed through five more generations of the same batch of Type I serum. The fifth serum culture was plated and several rough colonies were subcultured and these also were found to revert readily to the smooth form on being inoculated into mice.

Another protective Type I serum was then taken and a culture was started with a trace of blood from a mouse which had died from a Type I pneumococcal septicaemia. Four generations of serum cultures were made in succession, a night's incubation intervening between each. In the first two generations the culture grew in the form of a firm mass at the bottom of the serum; the third culture was partly granular and the fourth was quite diffuse. Plate cultures were made from the first, third and fourth generations and five colony cultures from each plate. These cultures were tested on mice, 27 mice with the first generation, six with the third and six with the fourth, the doses ranging from 1 c.c. of broth culture up to the deposit of 50 c.c.

All the strains, those from the first as well as the fourth serum cultures, were avirulent and none reverted to the smooth form.

It was clear that the second batch of Type I serum used was more efficient in producing attenuation than the first. In spite of eleven passages, the latter did not succeed in removing from the rough strain its ability to revert to the smooth on inoculation into mice.

A test was made to discover whether growth in the second more potent serum would further attenuate the readily reverting rough strain. After a night's incubation in the serum a plate was made and three rough colonies were subcultivated. These were tested on mice in subcutaneous doses of 1 c.c. of blood broth; two of the mice died and smooth colonies were recovered from the blood; the third mouse survived.

Thus the more active serum did not attenuate the rough strain so completely as it did the virulent capsulated pneumococcus sown from the blood. It is possible that pneumococci may to some extent become habituated to the action of the serum. For example, treatment with too low a concentration of protective antibodies seems to have induced the formation of a rough but reversible strain upon which the serum could no longer act.

The R culture of Type I most frequently used in the subsequent experiments was made from a rough colony (No. 3) grown from the first generation in the more powerful Type I serum referred to above.

### B. *Passage experiments through mice.*

The following is an example of many similar experiments which I have made to discover whether an avirulent R pneumococcus can be transformed into the virulent S form by growth in the body of the mouse. As a rule, the experiment has been started with the inoculation of one mouse and several lines of passage have subsequently developed, in only an occasional one of which has the transformation into the virulent form been effected. This irregularity of reversion has been a feature of the experiments where the culture has been passed through a succession of mice in small doses and by the intraperitoneal method of inoculation. Instances will be given later to show that a greater regularity may be attained when very large doses, viz. the centrifuged deposit of 50 to 100 c.c. of broth culture, are inoculated under the skin, though even then only a small proportion of the mice succumb to pneumococcal septicaemia where a thoroughly attenuated strain has been used.

This particular passage experiment was begun with rough Type II strains obtained as described on p. 125 and was continued along five separate lines. Pure line strains from single organisms were not used, but the preliminary tests on mice showed that the highly virulent pneumococcus had been eliminated by growth in the immune serum. As a further precaution, each of the five strains was plated and an isolated rough colony was grown in blood broth; this procedure was repeated six times and colonies from the final plates were made the starting point of the passage.

One of the five lines of passage is shown in Table IV.

Table IV.

Rough colony culture No. 1.
Deposit of 4 c.c. of broth culture inoculated subcutaneously

| | |
|---|---|
| M. 690<br>Killed 2 days | M. 691<br>Survived |
| Local lesion culture<br>Subcut. | |
| M. 713<br>Killed 2 days | |
| Local lesion culture<br>Rough colony<br>Subcut.<br>Deposit of 8 c.c. | |
| M. 723<br>Killed 2 days | M. 724<br>Survived |
| Local lesion culture<br>Rough colony<br>Subcut.<br>Deposit of 100 c.c. | |
| M. 747<br>Died 3 days<br>Smooth culture of T II<br>virulent in a dose of $10^{-8}$ | M. 746<br>Died 6 days |
| | Rough colonies from local lesion<br>Intrap. |
| | M. 801<br>Killed 9 days<br>P.M. culture<br>Nil |

The first two mice each received the centrifuged deposit of 4 c.c. of broth culture under the skin of the inguinal region. One mouse survived; the other was killed after two days, and from the tissue around the inguinal gland a blood plate and a blood broth culture were sown. Both cultures were pure, the plate showing rough colonies only, and the blood broth was inoculated into M. 713 in a dose of 0·5 c.c. Mouse 713 appeared well when killed two days later and a plate culture was made from the local lesion. A few small rough colonies grew and one of these was grown in broth. From this culture two mice were inoculated, each with the centrifugalised deposit of 8 c.c., under the skin of the inguinal region. One mouse, No. 723, was killed when well two days later and a plate culture from the local lesion yielded a few rough colonies with one of which the passage was continued. The fellow mouse was allowed to survive.

M. 723 culture was grown in 100 c.c. of broth and the centrifuged deposit was divided equally between two mice, both being inoculated subcutaneously. One mouse, No. 747, died in 3 days of pneumococcal septicaemia, the blood showing numerous capsulated diplococci. A smooth culture was obtained from the blood and this killed mice, inoculated intraperitoneally, in a dose of $10^{-8}$ c.c. of broth culture.

The fellow mouse, No. 746, died six days after inoculation; the blood was sterile and a few rough colonies were grown on a plate from the seat of inoculation. One of these colonies was grown in blood broth and a mouse inoculated intraperitoneally with 0·5 c.c. remained

well. The passage experiments with the other four rough colony cultures ended, as in the example described above, with the subcutaneous inoculation of two mice, each with the centrifuged deposit of 50 c.c. of broth culture. The results are as follows: in No. 2 passage both mice yielded smooth virulent cultures; Nos. 3 and 4 were like that first described, one mouse yielding rough colonies and the other smooth; in No. 5 the final cultures obtained from both mice were still rough and avirulent.

An intermediate rough culture in the fifth line of passage was taken and was again inoculated into two mice. One received subcutaneously the deposit of 66 c.c. of broth and the other the deposit of 33 c.c. The former died of pneumococcal septicaemia in three days, the blood yielding a smooth culture; the latter was killed after two days and rough colonies only were obtained from the local lesion.

In the above experiments it will be noted that the reversion from rough to smooth occurred in those mice which were inoculated subcutaneously with large amounts of culture. It seemed possible that this latter circumstance in affording a favourable nidus may have had more influence on the development of smooth characteristics than the transference from mouse to mouse. In order to test this view, four of the original rough cultures were inoculated subcutaneously into mice, each of which received the deposit of 50 c.c. Five survived and were healthy when killed ten days after inoculation; one died in three days and yielded a few rough colonies; only one died of septicaemia with numerous capsulated diplococci in the blood, from which a smooth culture of Type II was obtained. It may be mentioned that none of these cultures reverted when inoculated in a small dose, so it appears that a large dose favours the reversion, but does not produce it infallibly.

In the following experiment the passage was begun with two R strains of Type II which had been subcultivated for over a year and had been repeatedly both plated and tested on mice without producing any S colonies or showing any virulence for mice in moderate doses. Each culture was inoculated intraperitoneally into a mouse in a dose of 1 c.c. of broth culture. The mice were ill when killed the next day and plate cultures from the blood yielded sparse rough colonies. An R colony strain was made from each mouse (for convenience of description these have been designated A and B respectively); each was inoculated subcutaneously into three mice in doses of 1 c.c. of blood broth. All the mice survived for three days when they were killed apparently well and plate cultures were made from the seat of inoculation.

*Result of inoculation of rough culture A.*

Mouse 1 yielded a mixture of R and S colonies from the seat of inoculation. A subculture was made from each variety and tested on mice. The R strain failed to kill in a dose of 0·5 c.c. intraperitoneally; fatal septicaemia was produced by the S strain in a dose of 0·1 c.c. intraperitoneally and 0·5 c.c. subcutaneously, but not in smaller doses.

Mice 2 and 3 yielded R colonies only which were not virulent when inoculated into other mice.

*Result of inoculation of rough culture B.*

Mouse 1 gave only R colonies on the plate from the local lesion. A culture from one was inoculated subcutaneously in a dose of 1 c.c. into a mouse which died in two days from pneumococcal septicaemia; typical S colonies of Type II were grown from the blood.

Mouse 2 yielded a majority of R colonies and one S colony which agglutinated with Type II serum, an R colony was avirulent; the S colony killed mice in a subcutaneous dose of 0·1 c.c. but not in smaller doses.

Mouse 3 yielded R colonies which after two further passages in series through mice remained rough and avirulent.

This experiment shows that the ability of an attenuated R strain to revert to the virulent S form persists during prolonged periods of subculture in the R form.

C. *Inoculation of large doses of R pneumococci subcutaneously into mice.*

The following table gives the results of inoculating the deposits of broth culture of a single strain of attenuated R pneumococcus under the skin of mice.

| Mouse | Culture | Dose | Result |
|---|---|---|---|
| 744 | Rough Type II | Deposit of 50 c.c. | Died 3 days. R colonies only |
| 745 | ,, | ,, ,, ,, | ,, 3 ,, S colonies, Type II |
| 746 | ,, | ,, ,, ,, | ,, 6 ,, R colonies only |
| 747 | ,, | ,, ,, ,, | ,, 3 ,, S colonies, Type II |
| 748 | ,, | ,, ,, ,, | ,, 5 ,, S colonies, Type II |
| 749 | ,, | ,, ,, ,, | Killed 10 ,, R colonies only |
| 750 | ,, | ,, ,, ,, | Died 4 ,, S colonies, Type II |
| 751 | ,, | ,, ,, ,, | Killed 13 ,, Culture—nil |
| 752 | ,, | ,, ,, ,, | Died 3 ,, S colonies, Type II |
| 753 | ,, | ,, ,, ,, | Killed 13 ,, R colonies only |
| 759 | ,, | ,, ,, ,, | Died 2 ,, S colonies, Type II |
| 760 | ,, | ,, 30 ,, | Died 2 ,, S colonies, Type II |
| 761 | ,, | ,, 70 ,, | Survived ✓ |
| 762 | ,, | ,, 70 ,, | Died 1 day. S colonies, Type II |
| 763 | ,, | ,, 10 ,, | ,, 2 days. S colonies, Type II |
| 764 | ,, | ,, 20 ,, | Killed 6 ,, Culture—nil |

These experiments support the view expressed earlier that the subcutaneous inoculation of a mass of culture under the skin furnishes a nidus in which the R pneumococcus is able to develop into the virulent capsulated form and thence invade the blood stream. As mentioned earlier, passage experiments with smaller doses up to 1 c.c. of broth culture have also resulted in reversion to the virulent S form, but the occurrence is infrequent and irregular. The effect of the larger doses inoculated subcutaneously is more certain and in only 7 out of the 16 examples given has the R strain remained unaltered.

## Inoculation of Attenuated R pneumococci together with virulent S culture killed by heat.

### *Preliminary Experiments.*

The development of the virulent S form of pneumococcus from the R form inoculated in large doses under the skin of the mouse is no doubt favoured by the mass of culture forming a nidus. This protection from the normal defensive mechanism of the animal tissues cannot, however, be the sole factor in the production of the change, since the attenuated R pneumococcus may survive unaltered in the subcutaneous tissues for two or three weeks without any such protection.

Some R strains revert to the virulent type much more readily than others, and it is possible, as I have suggested above, that such strains may have retained in their structure a remnant of the original S antigen insufficient in ordinary circumstances to enable them to exert a pathogenic effect in the animal body. When a strain of this character is inoculated in a considerable mass under the skin, the majority of the cocci break up and the liberated

S antigen may furnish a pabulum which the viable R pneumococci can utilise to build up their rudimentary S structure. The amount of S antigen in an R strain, even one only partially attenuated, might not be very large, and it might happen that such an R strain did not liberate in sufficient concentration the stimulating or nutrient substances necessary to produce reversion. It appeared possible that suitable conditions could be arranged if the mass of culture was derived from killed virulent pneumococci, while the living R culture was reduced to an amount which, unaided, was invariably ineffective. There would be thus provided a nidus and a high concentration of S antigen to serve as a stimulus or a food, as the case may be.

A number of experiments on the above lines have been done and the results have shown quite conclusively that reversion of an attenuated R pneumococcus to its virulent prototype can be induced with much greater regularity than after inoculation of large amounts of the living R strain alone. The details of the procedure are given in the following example. A virulent culture of Type II was grown for a few hours in glucose broth, a fairly dense growth being produced, and was then killed by steaming at 100° C. The culture was concentrated by centrifuging and four mice were inoculated subcutaneously each with the deposit of 50 c.c. together with 0·5 c.c. of a living serum broth culture of R Type II. All four mice died in 3 to 5 days with numerous capsulated diplococci in the blood, cultures from which gave the typical agglutination reaction of a virulent Type II strain. A control experiment was made at the same time. Ten mice were inoculated subcutaneously each with the same amount of the living R strain as above together with the steamed deposit of 40 c.c. of broth culture of Type I. One of the mice died 2 days later from an infection with Gram-negative bacilli; the rest were killed healthy in 7–10 days. Cultures made from the tissues at the seat of inoculation remained sterile except in two mice, killed after 7 days, which yielded a few R colonies of pneumococci. This control experiment shows

(1) that the R pneumococcus of Type II remained attenuated in the absence of steamed virulent Type II, and

(2) that it was not assisted to regain virulence by the presence of the steamed Type I culture.

The reverse of the above experiment was then tried, the attenuated culture being the R form of Type I, but in this case there was found a difference in the results according to the temperature to which the heated virulent culture was raised. In an experiment with four mice steamed Type I (deposit of 70 c.c. of broth culture) together with 0·25 c.c. of the R cultures had no effect, all the mice being perfectly healthy when killed 17 days later. The same amount of Type I culture heated to 80° C. for one hour caused one mouse to die 10 days later and a virulent Type I culture was recovered from the blood; the other three mice were killed after 17 days and cultures from the seat of inoculation showed that the R pneumococci had perished.

The effect of Type I culture heated at 60° C. for two hours was then tried.

Six mice received subcutaneously the deposit of 60 c.c. of heated culture together with 0·25 c.c. of the rough Type I culture; five out of the six died of Type I pneumococcal septicaemia. In a repetition of the last experiment the same R culture of Type I in a dose of 0·25 c.c. was administered together with the deposit of 110 c.c. of virulent Type I culture which had been heated for two hours at 60° C. Ten mice were inoculated subcutaneously; one died within a few hours; the rest succumbed to pneumococcal septicaemia in 2–6 days and smooth Type I cultures were grown from the blood in every case.

A control experiment with the above rough Type I, inoculated plus the deposit of 100 c.c. of virulent Type II culture which had been steamed for 15 minutes, was negative. Ten mice were used and all were healthy when killed after 10–13 days. The R strain had disappeared except in one mouse where a few R colonies grew from the seat of inoculation.

In the preceding experiments the steamed Type II antigen exerted a specific influence since it caused only the R form of Type II to increase in virulence and had no effect on that of Type I. On the other hand, virulent Type I antigen appears to be injured by steaming at 100° C. and produces no effect either on the corresponding R form or upon the R form derived from Type II.

If the virulent Type I culture is heated only to 60° C. it very readily changes the R form of Type I into the virulent S form. But the lowering of the temperature at which the virulent culture is killed has other important results, as will be shown later. It may be mentioned now that the shorter the period during which the culture is heated to 60° C. (lower temperatures have not been tried), the more powerful and the less confined to its own type is the effect of the virulent antigen on the attenuated R form when the two are injected simultaneously into mice. For example, virulent cultures of Type I heated to 60° C. may cause the attenuated R strain of Type II to assume the capsulated S form in the animal body.

Short exposures at 60° C. introduce a risk which can be excluded when the cultures are killed by steaming at 100° C., the risk namely that some of the pneumococci in the culture may have survived the heating and be still viable. To ensure that the culture deposits have been uniformly heated, they have been enclosed in a sealed glass capsule and immersed in a large water-bath, the whole capsule being kept below the level of the water which has been carefully maintained at the required temperature. The heated deposits have been tested by incubation followed by plating and by the injection of large amounts subcutaneously into mice. These tests of viability have invariably been negative and exposure for so short a period as 15 minutes to a temperature of 60° C. appears to be sufficient to kill pneumococci. But the results of inoculating attenuated R strains into mice together with heated suspensions of virulent cultures of different types have been so remarkable as to raise the question whether the ordinary tests of viability are sufficiently comprehensive. It becomes necessary to consider whether heating to 60° C.

may produce resistant forms of pneumococci which do not multiply except when injected into a mouse together with a living attenuated pneumococcus. On account of the importance of the correct interpretation of the experiments which I am about to describe, I have given in detail a number of examples varying slightly in the experimental conditions and in the method of control.

As the heated S culture appears to be the determining factor, each series of experiments is headed by the particular type of virulent pneumococcus furnishing it. All inoculations designed to increase virulence or alter type have been done subcutaneously in mice.

*Inoculation experiments with heated virulent Group IV and attenuated R Type I and R Type II pneumococci.*

Table V shows that three different strains of Group IV killed by steam at 100° C., when injected into mice together with living attenuated R pneumococci derived from Type II by growth in homologous immune serum, caused the R form to revert to the virulent capsulated S form. R 4, Type II, *i.e.*

Table V.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Pn. 85, Group IV, steamed 20 mins. Dose = deposit of 60 c.c. of broth culture | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 405 | Died 4 days | S colonies, Type II |
| | | 406 | Killed 7 ,, | None |
| | | 407 | ,, 7 ,, | R colonies |
| | | 408 | Died 4 ,, | S colonies, Type II |
| Pn. 160, Group IV, as above | R 4, Type II as above | 409 | Killed 7 days | S colonies, Type II |
| | | 410 | Died 4 ,, | ,, ,, |
| | | 411 | ,, 4 ,, | ,, ,, |
| | | 412 | ,, 3 ,, | ,, ,, |
| II B, Group IV, as above | R 4, Type II as above | 413 | Died 3 days | S colonies, Type II |
| | | 414 | ,, 2 ,, | ,, ,, |
| | | 415 | ,, 3 ,, | ,, ,, |
| | | 416 | Killed 7 ,, | R colonies |
| None | R 4, Type II. Doses = 0·75, 1·0, 1·0 c.c. of blood broth culture | 462 | Killed 19 days | None |
| | | 463 | ,, 19 ,, | ,, |
| | | 464 | ,, 19 ,, | ,, |

rough colony 4, Type II, which has been used throughout these experiments is so much attenuated as never to kill mice in doses of 1·0 c.c. of blood broth culture. The three control mice remained well and were killed 19 days after inoculation. As mentioned earlier, all the inoculation experiments to increase virulence have been done subcutaneously and it has been the custom in the case of mice killed when apparently well to make plate cultures from the tissues at the seat of inoculation, viz., the right groin. Generally the plates are either sterile or purely pneumococcal, but care must be taken should the inoculated material cause the skin to ulcerate. Extraneous organisms are usually staphylococci which cause no difficulty. There were additional controls to the above experiments. These have not been given in the table, but they show that the stimulus to reversion of the R strain was not possessed by

steamed Type III culture (deposit of 60 c.c. for each of 12 mice) or by scarlatinal streptococci (4 mice).

Table VI.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Pn. 85, Group IV, heated 2 hours at 60° C. Dose = deposit of 50 c.c. of broth culture | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 791 | Killed 7 days | None |
| | | 792 | Died 3 ,, | S colonies, Type II |
| | | 793 | ,, 3 ,, | ,, ,, |
| Pn. 85, Group IV, as above | R 6, Type I, as above | 794 | Killed 16 days | None |
| | | 795 | ,, 16 ,, | ,, |
| | | 796 | ,, 16 ,, | ,, |
| Pn. 160, Group IV, as above | R 4, Type II, as above | 773 | Died 2 days | S colonies, Type II |
| | | 774 | ,, 3 ,, | ,, ,, |
| | | 775 | ,, 2 ,, | ,, ,, |
| Pn. 160, Group IV, as above | R 6, Type I, as above | 776 | Killed 15 days | None |
| | | 777 | ,, 15 ,, | ,, |
| | | 778 | ,, 15 ,, | ,, |
| II B, Group IV, as above | R 4, Type II, as above | 785 | Died 3 days | S colonies, Type II |
| | | 786 | ,, 3 ,, | ,, ,, |
| | | 787 | ,, 3 ,, | ,, ,, |
| II B, Group IV, as above | R 6, Type I, as above | 788 | Killed 15 days | None |
| | | 789 | ,, 15 ,, | ,, |
| | | 790 | ,, 15 ,, | ,, |

Table VI confirms the conclusions drawn from the experiments in Table V, that Group IV S antigen enables attenuated Type II pneumococci to become virulent in the mouse.

In addition it shows that Group IV antigens, contained in three virulent strains, after heating to 60° C. for an hour on two occasions, did not produce reversion of the R form of Type I; the mice inoculated remained well and no culture could be obtained from the seat of inoculation 15–16 days later.

The latter part of the experiment, which shows that an attenuated R culture of Type I is not increased in virulence through the influence of heated S antigens of Group IV, was repeated with identical results. The same three Group IV strains, heated at 60° C., were injected in doses of 100 c.c. of broth culture deposit together with a different rough culture, R 3, Type I (this signifies rough colony 3, derived from Type I and this strain has been most frequently employed in these experiments). All the mice, a total of 21, were healthy when killed 22–28 days later.

These experiments may be summarised as follows.

Group IV virulent pneumococci, killed either by heating to 60° C., or by steam at 100° C., when injected in large quantities of culture deposit into mice together with an attenuated R strain of Type II caused the latter to revert to the capsulated virulent form and set up fatal septicaemia in the mice.

Under similar conditions three Group IV cultures failed to increase the virulence of attenuated R strains derived from Type I.

*Inoculation experiments with heated virulent Type I culture and attenuated R strains of Types I and II.*

*Conversion of R Type II into S Type I.* In the experiment in Table VII two out of eight mice injected with heated virulent Type I culture together with an attenuated R culture derived from Type II died of pneumococcal septicaemia and yielded pure S colonies of Type I from the blood; plates from the lesions at the seat of inoculation showed a mixture of R and S colonies.

Table VII.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Type I heated 2 hours at 60° C. Dose = deposit of 50 c.c. of broth culture | None | 641 | Killed 5 days | None |
| | ,, | 642 | ,, 6 ,, | ,, |
| | ,, | 643 | ,, 6 ,, | ,, |
| | ,, | 644 | ,, 6 ,, | ,, |
| As above | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 645 | Died 3 days | S colonies, Type I |
| | | 646 | Killed 5 ,, | R cols. from local lesion |
| | | 647 | ,, 6 ,, | ,, ,, |
| | | 648 | ,, 6 ,, | ,, ,, |
| As above | R 4, Type II, grown in the heated Type I deposit. Dose = 0·36 c.c. | 649 | Killed 5 days | R cols. from local lesion |
| | | 650 | Died 4 ,, | S colonies, Type I |
| | | 651 | Killed 6 ,, | None |
| | | 652 | ,, 6 ,, | One R colony |

The remaining six mice were killed, apparently healthy, after 5–6 days and plate cultures made from the subcutaneous seat of inoculation either produced only R colonies or remained sterile.

The four mice which received the heated Type I culture alone were well when killed 5–6 days later, and plates made from the subcutaneous tissues at the seat of injection remained sterile.

The rough attenuated Type II culture has apparently in two instances been changed into a virulent Type I. The most obvious alternative to this presumption is that a few Type I pneumococci in the heated culture remained viable. It is therefore desirable to give in detail the cultural tests and the treatment to which the virulent culture had been submitted. After heating for one hour at 60° C. a large loopful of suspension was sown on blood agar plates. The tube containing the thick suspension (the deposit of 600 c.c. concentrated by centrifuging to 12 c.c.) was resealed and again heated to 60° C. for one hour. The suspension was then incubated at 37° C. overnight and plated the next day. All the above plates remained sterile. In order to show that the thick suspension would not inhibit the growth of viable pneumococci 4 c.c. of it were sown with the R strain of Type II, incubated overnight and plated; a profuse growth of R colonies only was obtained. This culture in the suspension was used for the last four mice in the table.

In view of these precautions, I think it may be assumed that the heated suspension contained no viable Type I pneumococci. The R strain of Type II, although not tested alone on this occasion, has been used throughout these

experiments and numerous tests have shown that it is a pure attenuated rough culture.

A second experiment (see Table VIII) on the same lines was made and the cultural tests of viability of the heated culture were as rigid as possible. The thick suspension of the concentrated broth culture after heating was distributed in ten small tubes so that each tube contained the deposit of 100 c.c. and to each of them was added 1 c.c. of sterile bovine serum. The mixtures were incubated overnight and subcultures were made the next day both upon fresh blood agar plates and in blood broth; the latter were also plated after incubation at 37° C. The cultures from every tube remained sterile.

Table VIII.

| Killed S pneumococci | Living R pneumococci | No. of mice | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Type I heated 3 hours at 60° C. Dose = deposit of 50 c.c. in salt solution | None | 4 | Killed 7 days | None |
| Type I as above. Dose = deposit of 100 c.c. | None | 2 | Killed 7 days | None |
| Type I as above. Dose = deposit of 50 c.c. | R 4, Type II. Dose = 0·2 c.c. of blood broth culture | 8 | Killed 7 days | R colonies from 4 and none from the rest |
| None | R 4, Type II. Dose = 0·5 c.c. | 2 | Killed 13 days | None |
| Type I as above. Dose = deposit of 100 c.c. | R 4, Type II. Grown in heated culture | 2 | Killed 13 days | None |
| Type I deposit of 100 c.c. (bovine serum not removed) | R 4, Type II. Dose = 0·25 c.c. | 1 | Died 3 days | S colonies, Type I |

Seven of the tubes of suspension were centrifuged; the supernatant serum was removed and replaced by 0·5 c.c. of salt solution. Eight mice were inoculated subcutaneously each with the deposit of 50 c.c. of the heated culture together with 0·2 c.c. of blood broth culture of the living R strain of Type II. Six mice were similarly injected with the heated culture alone, four receiving the deposit of 50 c.c. and two the deposit of 100 c.c. of the broth culture.

All of the above mice were killed seven days later; cultures made from the seat of inoculation were completely negative in the case of the control mice and all of those receiving the mixtures except four which yielded a few R colonies only.

*Failure of attempt to induce reversion in vitro.* Two tubes containing heated suspension (the concentrated deposit of 100 c.c.) in serum were used as culture media. They were sown with the R strain of Type II to ascertain if it could be changed into the S form *in vitro*. After incubation overnight plates were made and the tubes were then centrifuged. The supernatant serum was pipetted off, leaving the deposit to which was added a fresh quantity of sterile bovine serum. This procedure was repeated eight times and the final as well as the intermediate plate cultures yielded only R colonies.

The whole of the two deposits, each containing the heated Type I pneumococci from 100 c.c. plus the growth of the R strain, were inoculated into two mice. These were killed 13 days later and found to be healthy.

*Importance of the presence of all the products in the smooth culture.* There was one tube of Type I suspension remaining from which the bovine serum added a week earlier had not been removed. This was inoculated into a mouse together with 0·25 c.c. of an 18-hour old blood broth culture of the R II strain. The mouse was killed when ill 3 days later and smooth virulent Type I pneumococci were grown from the blood and from the subcutaneous seat of inoculation.

The negative result in the first part of the experiment may have been due to the removal of some important substance from the heated culture deposit. As mentioned above, 1 c.c. of sterile bovine serum was added to each tube of deposit in order to make it a favourable medium for the growth of any viable pneumococci possibly remaining. The total bulk, being now over 1·5 c.c. and being mainly serum, was too great for subcutaneous inoculation into a mouse; so each tube was centrifuged, the supernatant serum was removed and salt solution was substituted.

The experiment shown in Table IX was done to test the hypothesis suggested above.

Table IX.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Type I heated 1½ hours at 60° C. Dose = deposit of 70 c.c. in salt solution | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 817 | Killed 13 days | None |
| | | 818 | ,, 13 ,, | ,, |
| | | 819 | Died 3 ,, | ? S colonies, Type I |
| | | 820 | Killed 13 ,, | None |
| | | 825 | ,, 13 ,, | ,, |
| Supernatant bovine serum removed from the above deposits | As above | 826 | Killed 16 days | None |
| | | 827 | ,, 16 ,, | ,, |
| | | 828 | ,, 16 ,, | ,, |
| | | 829 | ,, 16 ,, | ,, |
| | | 830 | ,, 16 ,, | ,, |
| Type I heated as above. Dose = deposit of 70 c.c. with bovine serum | As above | 821 | Died 4 days | S colonies, Type I |
| | | 822 | ,, 3 ,, | ,, ,, |
| | | 823 | Killed 13 ,, | None |
| | | 824 | ,, 13 ,, | ,, |

A glucose broth culture of Type I, after concentration by centrifuging, was heated to 60° C. in a sealed tube for half an hour and again for one hour. The whole of the culture (700 c.c. reduced to 5 c.c.) was distributed in small tubes, 0·5 c.c. in a tube, and to each was added 0·5 c.c. of sterile bovine serum. The tubes were incubated overnight at 37° C. and each was plated; they were plated again after a second incubation. All were sterile. The tubes were then divided into two sets. Five were centrifuged and the deposit was separated from the supernatant serum broth. Four of the remaining were retained whole.

To each of the 14 tubes thus obtained 0·25 c.c. of the attenuated R strain was added and the mixtures were injected into mice.

Two of the four mice which received the whole culture died of pneumococcal septicaemia and yielded S colonies of Type I.

One of the five mice inoculated with the deposit resuspended in salt solution died and numerous small colonies, not definitely smooth, grew from the blood. These colonies, however, formed firm masses with Type I serum and one colony subculture killed a mouse from which typical S colonies of Type I were obtained.

There is a strong suggestion that the whole deposit is more effective in converting the R strain into a virulent form than the deposit from which the serum was removed after incubation, *i.e.* the deposit which had been washed with serum. The supernatant fluid, however, did not contain any substance in sufficient concentration to increase the virulence of the R culture. Although the experiment is not decisive, it rather indicates that the essential material for the building up of a virulent form from the R form may be associated with the capsule of the pneumococcus and may be to some extent washed off.

*Conditions affecting the efficacy of the S antigen in inducing reversion.* It is certain that the efficacy of the S antigen in providing the conditions necessary for recovery of virulence is variable and, for example, differences in temperature and period of heating may exert considerable influence, as will be seen from the following experiments.

In Table X are given the results of heating the S culture at 60° C. for different periods, ranging from 15 minutes to 50 minutes.

Each heated deposit alone was injected into 3 mice, while 2 mice received

Table X.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from mouse |
|---|---|---|---|---|
| Type I. Dose = deposit of 100 c.c. glucose broth culture heated 15 mins. at 60° C. | None | 978 | Killed 13 days | None |
| | ,, | 979 | ,, 13 ,, | ,, |
| | ,, | 980 | ,, 13 ,, | ,, |
| As above | R 4, Type II. Dose = 0·25 c.c. | 994 | Died 2 days | S colonies, Types II and I |
| | | 995 | ,, 3 ,, | S colonies, Type I |
| Type I heated 25 minutes at 60° C. | None | 981 | Killed 13 days | None |
| | ,, | 982 | ,, 13 ,, | ,, |
| | ,, | 983 | ,, 13 ,, | ,, |
| As above | R 4, Type II | 996 | Died 3 days | S colonies, Type I |
| | | 997 | ,, 2 ,, | ,, Type II |
| Type I heated 40 minutes at 60° C. | None | 984 | Killed 13 days | None |
| | ,, | 985 | ,, 13 ,, | ,, |
| | ,, | 986 | ,, 13 ,, | ,, |
| As above | R 4, Type II | 998 | Died 2 days | S colonies, Type II |
| | | 999 | ,, 2 ,, | ,, Type I |
| Type I heated 50 minutes at 60° C. | None | 987 | Killed 13 days | None |
| | ,, | 988 | ,, 13 ,, | ,, |
| | ,, | 989 | ,, 13 ,, | ,, |
| As above | R 4, Type II | 1000 | Killed 12 days | None |
| | | 1 | Died 3 ,, (Pn. in blood) | Culture overgrown |

the combined inoculation. All the control mice were killed 13 days later and plate cultures from the subcutaneous seat of inoculation remained sterile. Culture tests also showed the heated culture to be sterile.

On the other hand, all the mice, except one, inoculated with the attenuated strain in addition died of pneumococcal septicaemia within 3 days. The mouse which survived had received the deposit heated for 50 minutes. The fellow mouse died and the culture from the blood became overgrown. The infection was most probably due to Type II pneumococci, since microscopical examination of the blood showed diplococci with well-marked capsules. (As a rule a Type I pneumococcus appears in the mouse's blood in short chains and the capsules are rarely as large and well stained as those of Type II pneumococci.)

The S culture heated for 15, 25 and 40 minutes had almost identical effect on each pair of mice, one yielding a virulent Type I culture and the other a virulent Type II. From one mouse injected with the 15 minutes heated deposit Type II colonies were grown from the blood, while on the plate from the local lesion among the majority of translucent Type II colonies a single slightly opalescent colony was identified as Type I.

The above results are more striking than the examples already given with cultures heated at 60° C. for an hour or more and there is an indication of a falling off as the period of heating approaches the hour.

An experiment was therefore made to compare at the same time cultures heated for over and under an hour.

A virulent Type I culture was grown for a few hours in 2 litres of glucose broth. It was then centrifuged and the deposit was collected into two sealed tubes each containing 10 c.c. Both were heated in a water-bath at 60° C. for half an hour. The next day one was heated to the same temperature as before for a further period of $2\frac{1}{2}$ hours. The tubes were then opened and the thick suspension was distributed into tubes in 1 c.c. quantities to each of which was added 0·25 c.c. of blood broth. The tubes were incubated overnight and plated the next day. One had become contaminated with a bacillus; the rest were sterile. The heated cultures alone were not tested on mice. Ten mice were inoculated with the culture heated for 3 hours and nine with the portion heated for half an hour. Each mouse received the deposit of 100 c.c. together with 0·25 c.c. of the attenuated R strain of Type II.

The results showed very definitely that there was a considerable difference in character between the two differently heated suspensions.

Of the ten mice which received the 3-hour heated culture three died of septicaemia and S colonies of Type II were grown from the blood. One died prematurely (3 days) and a few R colonies were grown both from the blood and from the local lesion. The rest were killed in 6–7 days and R colonies only were obtained from the seat of inoculation.

Of the nine mice which received the $\frac{1}{2}$-hour heated culture five died of septicaemia in 3–6 days and S colonies of Type I were grown from the blood.

One mouse died in 4 days; the blood yielded S colonies of Type II, but among these was observed a colony a little whiter than the rest. A subculture of the latter inoculated into a mouse intraperitoneally produced septicaemia; a culture and the peritoneal washing gave positive agglutino-precipitation with Type I serum. The seventh mouse died in 4 days of Type II septicaemia. The remaining two were killed in 6 days; they showed nothing microscopically in the blood and a few R colonies only were grown from the seat of inoculation.

*The question of intermediate stages in the transformation of type.* An attempt has been made, from time to time, to ascertain whether, if it is the case that the virulent S form is built up from the R form, the change is gradual, *i.e.* whether the R form before developing the virulent S form of another type passes through a stage when, though still R, it resembles the R of the new type. The only way to differentiate with certainty between the R strain derived from Type I and the R strain derived from Type II is by producing reversion to the S form of the original type without the assistance of any heated S antigen. The experiment just described offered a suitable occasion for testing this point. An R colony from the local lesion from one of the above mice which died of Type I septicaemia was grown in 100 c.c. of glucose broth. The centrifuged deposit was divided equally between two mice by subcutaneous inoculation. One died of Type II septicaemia, the other remained unaffected. The R colony chosen was therefore the same as that inoculated, viz. the R colony of Type II. Colonies were also taken from five of the mice in which no virulent S form had made its appearance and they were treated as above. Two of the colony cultures, inoculated in 50 c.c. doses, caused Type II septicaemia in one of each pair of mice; all the other mice survived.

Positive evidence of an intermediate R form between the attenuated Type II and the possibly newly formed virulent Type I thus fails.

*Further experiments on the destructive effect of heat on Type I S antigen.* The effect of heating the S culture to temperatures above 60° C. has been given some attention. It has already been noted that Type I virulent culture after exposure to 100° C. loses the property possessed by cultures heated to 60° C. of restoring the virulence of the R strain of Type I inoculated at the same time and does not cause the appearance of S cultures of Type I when injected along with an attenuated R strain of Type II.

The results of injecting Type I culture heated for 15 minutes at temperatures ranging from 60° C. to 80° C. are given in Table XI. Only the deposit heated at 60° C. was tested on mice for the presence of viable pneumococci. Four mice received each the deposit of 65 c.c. of glucose broth and were killed 10 days later. Plates made from the seat of inoculation were sterile; the subcutaneous tissue from the groin of each mouse was removed, emulsified and injected into a second mouse intraperitoneally; all these mice survived. It seems clear that no viable pneumococci remained in the culture heated to 60° C. for 15 minutes.

Table XI.

| Killed S pneumococci | Living R pneumococci | No. of mice | Result | Type of culture obtained from the mouse |
|---|---|---|---|---|
| Type I heated at 60° C. for 15 minutes. Dose = deposit of 65 c.c. of culture | None | 4 | Killed 10 days | None |
| Type I as above. Dose = deposit of 30 c.c. | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 2 | Died 3 and 5 days | S colonies of Type I from blood |
| Type I heated at 65° C. for 15 minutes. Dose = deposit of 65 c.c. | R 4, Type II, as above | 4 | Killed 10 days | None |
| Type I heated at 70° C. as above | R 4, Type II, as above | 4 | Killed 10 days (2)<br>Died 4·5 days (2) | None (2)<br>S colonies of Type II (i)<br>S colonies of Types I and II (1) |
| Type I heated at 75° C. as above | R 4, Type II, as above | 4 | Killed 10 days | None |
| Type I heated at 80° C. as above | R 4, Type II, as above | 4 | Killed 10 days (3)<br>Died 3 „ (1) | None (3)<br>R colonies only (1) |

Both mice inoculated with the R strain of Type II together with the deposit of 30 c.c. of this broth culture of Type I heated at 60° C. developed Type I septicaemia.

The results with the R Type II plus Type I culture heated at 70° C. were interesting. Two mice died of pneumococcal septicaemia. S colonies of Type II were grown from the blood of one and S colonies of Type I from the other. The local lesion of the latter mouse was plated and among a majority of R colonies a few S colonies grew, two of which were tested and found to be Type II.

From the remaining mice, most of them being killed healthy, no S cultures were obtained.

The Type I cultures heated at different temperatures were also tested on mice together with an R strain derived from Type I; the dose of heated culture was only half the amount used in combination with the R strain of Type II. All the mice which received the culture heated at 65° and 70° C. respectively died of Type I septicaemia. At 75° C. the culture was effective in causing reversion to the S form of Type I in two out of three mice, whilst the culture heated at 80° C. produced no effect, the mice remaining well and the R pneumococci having disappeared from the seat of inoculation. The destructive effect of the increased temperature on the Type I antigen is thus exhibited as in the preceding experiments.

These experiments may be summarised as follows.

The injection of heated virulent S culture of Type I into the subcutaneous tissues of mice together with an attenuated R strain derived from Type II apparently results in the conversion of the latter into a virulent S culture of Type I or of Type II. Cultures heated for 15 minutes at 60° C. are more

effective in producing the transformation than cultures heated for longer periods at 60° C. or at higher temperatures.

The chances of an R strain of Type II reverting to its original S form or being converted into the S form of Type I after inoculation together with virulent Type I culture heated at 60° C. for 15 minutes appear to be about equal.

At 80° C. the active substance in the Type I pneumococcus is so much altered that the heated culture causes neither reversion of the R form of Type I to its S form nor transformation of the R form of Type II into the S form of Type I.

Cultures of Type I heated at 70° C. and 75° C. respectively are still effective in producing reversion of the R form of Type I, but transformation of the R form of Type II is rarely brought about by cultures heated higher than 60° C. One positive result with culture heated at 70° C. for 15 minutes was obtained.

Neither reversion to the S form nor transformation of type has been obtained *in vitro*.

*Inoculation experiments with heated virulent Type III culture and* (1) *an attenuated R strain derived from Type I, and* (2) *an attenuated R strain of Type II.*

The Type III culture used in the experiments shown in Table XII was grown for a few hours in a flask of glucose broth which on removal from the incubator was immersed in a water-bath at 60° C. for one hour to arrest

Table XII.

| Killed S pneumococci | Living R pneumococci | No. of mouse | Result | Type of culture obtained from the mouse |
|---|---|---|---|---|
| Type III heated 2 hours at 60° C. Dose = deposit of 100 c.c. of glucose broth culture | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 869 | Killed 14 days | None |
| | | 870 | ,, 14 ,, | ,, |
| | | 871 | Died 7 ,, | S colonies, Type III |
| | | 872 | ,, 10 ,, | ,, ,, |
| | | 873 | Killed 14 ,, | ,, ,, |
| | | 874 | ,, 14 ,, | None |
| | | 875 | Died 2 ,, | S colony, Type III |
| | | 876 | ,, 8 ,, | S colonies, Type III |
| Type III, as above | R 3, Type I. Dose = 0·25 c.c. of blood broth culture | 877 | Killed 14 days | None |
| | | 878 | ,, 14 ,, | ,, |
| | | 879 | ,, 14 ,, | S colonies, Type III |
| | | 880 | ,, 14 ,, | None |
| | | 881 | ,, 14 ,, | ,, |
| | | 882 | ,, 14 ,, | ,, |
| | | 883 | ,, 14 ,, | ,, |
| | | 884 | ,, 14 ,, | ,, |
| Type III steamed at 100° C. for 12 minutes. Dose = deposit of 100 c.c. | R 4, Type II. Dose = 0·25 c.c. | 885 | Died 3 days | S colonies, Type II |
| | | 886 | Killed 14 ,, | None |
| | | 887 | ,, 14 ,, | ,, |
| | | 888 | Died 3 ,, | S colonies, Type II |
| Type III, as above | R 3, Type I. Dose = 0·25 c.c. | 889 | Killed 14 days | R colonies only |
| | | 890 | Died 12 ,, | None |
| | | 891 | Killed 14 ,, | ,, |
| | | 892 | ,, 14 ,, | R colonies and one S colony |

autolysis. On the following day the 2400 c.c. of broth were concentrated by centrifuging to 24 c.c.; 16 c.c. were placed in a sealed tube and heated for one hour at 60° C., and the remaining 8 c.c. were steamed at 100° C. for a full 12 minutes.

In this instance mice were not injected with the heated culture alone.

(*a*) *Heated to* 60° *C.* Eight mice were inoculated with the R strain of Type I and eight with the R strain of Type II, both accompanied by a dose equivalent to 100 c.c. of Type III S culture heated to 60° C. It will be seen from Table XII that the watery colonies of Type III appeared more frequently in the mice inoculated with the attenuated R strain of Type II than in those which received the R strain of Type I, viz. five times in the former and once in the latter. This fact lends some support to the view that the particular type of R strain is the important factor in the production of the S colonies of Type III. Incidentally it is further evidence against the hypothesis that viable Type III pneumococci persisted in the culture after heating.

The low virulence of these newly formed Type III pneumococci, as one might term them, is noteworthy. The mouse inoculated with the R strain of Type I and the heated Type III culture was apparently well when killed 14 days later, and the S colonies came to light on the plate made from the subcutaneous seat of inoculation. These S pneumococci, which were in pure culture, were certainly multiplying in the local lesion and, though evidently of low virulence, might ultimately have caused the death of the mouse. Some of the Type III pneumococci from the mice inoculated with the R strain of Type II showed more virulence, since three out of the five positive mice died with pneumococcal septicaemia in 7–10 days. Mouse 875, which probably died prematurely from shock, yielded a single S colony among numerous R colonies from the seat of inoculation; the blood culture was contaminated. The fifth mouse, 873, was, like that in the first mentioned series, perfectly well when killed, and S colonies were grown from the seat of inoculation.

The large watery colonies of Type III on blood agar plates were very typical in appearance, and, in addition, the blood smears from the mice which died showed the characteristic picture of round cocci with large well-stained capsules. I have had to rely for the identification of Type III pneumococci on the above appearances in conjunction with negative agglutination reactions to both Types I and II sera, since, in spite of several trials, I have not recently been able to prepare a serum which gives the characteristic agglutination reaction with a virulent Type III culture.

(*b*) *Heated to* 100° *C.* The steamed Type III culture injected with the attenuated R strain of Type II caused the latter to revert to the virulent S form in two out of four mice.

An interesting result was obtained in one of the four mice which received the R strain of Type I together with the steamed Type III culture. The plate culture from an abscess at the seat of inoculation produced numerous small apparently rough colonies and one typical smooth disc-shaped colony.

Subcultures from the latter were virulent for mice; pneumococci multiplied in the capsulated form in the blood, and cultures from the blood yielded typical smooth discs. I was unable to identify the strain as Type III, since the typical watery colony was not produced; also no agglutination was obtained with Types I and II sera or with any of the available group IV sera.

In either case, whether it is a Group IV strain or a Type III, the result is important enough to encourage further experiments with steamed culture.

One must however bear in mind the possibility of accidental contamination, especially in view of a somewhat similar circumstance which is recorded in the description of the next experiment.

A virulent Type III culture in glucose broth was heated for 3 hours at 60° C. in a sealed tube and was injected subcutaneously into seven mice together with an R strain of Type II. The dose of the former was the deposit of 70 c.c. and of the latter 0·2 c.c. of blood broth. Four mice died in 4–7 days of pneumococcal septicaemia and watery colonies of Type III were grown from the blood; the remaining three mice were killed in 7 days and in each case similar colonies were grown from the seat of inoculation. The above was an exceptional result and fortunately an equal number of mice were injected with the same dose of the heated Type III culture alone. All the latter seven mice were killed on the 7th day after injection and cultures were made from the seat of inoculation. All the plates were sterile with one exception in which a pneumococcus colony appeared.

(In view of this disconcerting result in one of the control mice, I will describe in detail the procedure in making cultures from the seat of injection.

The mouse is pinned out and the abdominal skin is seared with a hot iron, but not exactly over the seat of injection. Through the seared area an incision is made with a sterile knife and the edge of the skin is grasped with a pair of forceps. The skin, held firmly, is reflected back until the seat of injection is exposed and is then pinned down. A fresh pair of sterile instruments is used to scrape the subcutaneous tissues and usually the gland in the groin is removed as well. The material is placed in a small tube with a few drops of broth in which it is rubbed up. A loopful of the fluid is spread on a plate and to the remainder a little blood broth is added. If the next day after incubation the fluid culture shows on microscopical examination of smears any diplococci it also is plated.)

In the case of the mouse referred to above there were no diplococci in the tissue fluid after incubation and the plate made subsequently from it produced no growth. The plate direct from the tissue emulsion was sterile except for a single smooth disc shaped colony which was bile soluble and virulent for mice. At no time did this strain produce watery colonies like Type III and there was no agglutination with Types I and II sera.

This result occurred some three months before the writing of this article and in spite of numerous tests designed to reveal the presence of any viable pneumococci in heated cultures I have never again found any evidence of viability either by culture or through the mouse, even in cultures heated at 60° C. for so short a period as 15 minutes.

On the whole I am inclined to think that the pneumococcus responsible for the colony fell on the plate while it was being spread.

Still it is often useful to record unexpected occurrences when there is no absolutely certain explanation. A result which appears to the worker concentrated on a particular issue to be a regrettable flaw in his working may be significant to another considering the subject from a different point of view.

These experiments may be summarised as follows:

The injection of S culture of Type III, heated at 60° C. for two periods

of one hour each, along with living R strains derived from Type I or Type II results in the appearance of an S pneumococcus of Type III.

This transformation of type occurs more frequently with the R form of Type II than with the R form of Type I.

The newly formed strains of Type III sometimes kill the mice from septicaemia in 7–10 days and at other times are only discovered at the seat of inoculation when the mouse is killed, apparently well, 14 days after inoculation.

*Inoculation experiments with heated virulent Type II culture and different attenuated R colony strains of Type I.*

The results of inoculating attenuated R strains of Type I together with heated Type II culture are shown in Table XIII. A comparison is made between different R colonies (developed on a plate from a virulent Type I culture grown in homologous immune serum).

The R strains were also inoculated into mice (1) alone, (2) together with

Table XIII.

| Killed S pneumococci | Living R Type I pneumococci | No. of mouse | Result | Type of culture obtained from the mouse |
|---|---|---|---|---|
| Type II heated 2 hours at 60° C. Dose = deposit of 90 c.c. | R colony 1 | 724 | Killed 12 days | None |
| | ,, 1 | 725 | ,, 12 ,, | ,, |
| | ,, 2 | 726 | Died 11 ,, | S colonies, Type II |
| | ,, 2 | 727 | ,, 10 ,, | ,, ,, |
| | ,, 3 | 728 | ,, 4 ,, | ,, ,, |
| | ,, 3 | 729 | ,, 4 ,, | ,, ,, |
| | ,, 4 | 730 | Killed 11 ,, | ,, ,, |
| | ,, 4 | 731 | Died 4 ,, | ,, ,, |
| | ,, 5 | 732 | Killed 13 ,, | ,, ,, |
| | ,, 5 | 733 | ,, 6 ,, | ,, ,, |
| | ,, 6 | 734 | Died 9 ,, | ,, ,, |
| | ,, 6 | 735 | Killed 13 ,, | None |
| Type III heated 2 hours at 60° C. Dose = deposit of 80 c.c. | R colony 1 | 736 | Killed 11 days | None |
| | ,, 1 | 737 | ,, 11 ,, | ,, |
| | ,, 2 | 738 | ,, 9 ,, | ,, |
| | ,, 2 | 739 | ,, 11 ,, | ,, |
| | ,, 3 | 740 | ,, 9 ,, | ,, |
| | ,, 3 | 741 | ,, 11 ,, | ,, |
| | ,, 4 | 742 | ,, 10 ,, | S colonies, Type III |
| | ,, 4 | 743 | ,, 11 ,, | None |
| | ,, 5 | 744 | ,, 10 ,, | ,, |
| | ,, 5 | 745 | ,, 11 ,, | ,, |
| | ,, 6 | 746 | ,, 11 ,, | ,, |
| | ,, 6 | 747 | ,, 9 ,, | ,, |
| Type II heated 3 hours at 60° C. Dose = deposit of 90 c.c. | R colony 1 | 748 | Killed 12 days | None |
| | ,, 1 | 749 | ,, 12 ,, | ,, |
| | ,, 2 | 750 | ,, 13 ,, | ,, |
| | ,, 2 | 751 | ,, 6 ,, | S colonies, Type II |
| | ,, 3 | 752 | Died 3 ,, | ,, ,, |
| | ,, 3 | 753 | Killed 13 ,, | None |
| Type III heated 3 hours at 60° C. Dose = deposit of 80 c.c. | R colony 4 | 754 | Killed 10 days | None |
| | ,, 4 | 755 | ,, 9 ,, | S colonies, Type III |
| | ,, 5 | 756 | ,, 9 ,, | None |
| | ,, 5 | 757 | ,, 9 ,, | ,, |
| | ,, 6 | 758 | ,, 11 ,, | ,, |
| | ,, 6 | 759 | ,, 10 ,, | ,, |

heated Type I culture, and (3) with heated Type III culture; the results with the last only appear in the table.

The R strains were each tested subcutaneously on two mice in a dose of about 1 c.c. of blood broth culture; all the mice were well when killed in 9–16 days and no pneumococci were recovered from the seat of inoculation.

The virulent cultures were killed by heating for two hours at 60° C.; in the case of Types II and III, cultures heated for 3 hours were also used. As a test of sterility the Types II and III cultures (heated 2 hours) were injected into two mice. Each mouse received subcutaneously the deposit of 130 c.c. of glucose broth; both remained well and were killed 16 days later. Cultures from the seat of inoculation remained sterile; in one of the two mice the culture was made from an encapsulated abscess which had formed under the skin and still contained fairly well staining diplococci.

From the majority of the mice inoculated with the mixture of the above heated culture and the living R strains of Type I, virulent S colonies of Type II were obtained. In the case of the R colony 1 both mice were negative while only one of the pair inoculated with R colony 6 yielded S colonies of Type II.

It is interesting that of all the mice inoculated with the twice heated Type I culture together with the R strains only the one which received R colony 1 failed to develop fatal Type I septicaemia. This result certainly suggests that R strains may differ in their suitability for mutation in the same way as they differ for reversion experiments.

There is a similar indication in the experiments with Type III heated culture. Only one R strain, R colony 4, yielded the watery colonies of Type III and the positive results occurred with the suspension heated for two hours and three hours respectively.

It will be noticed that the heating for an additional hour has lowered the proportion of positive results with the killed virulent Type II culture. The experiments with heated Type II culture and attenuated R strains derived from Type I have not always been so successful as the above in producing an apparent change of type, as will be seen from the following example.

A suspension of virulent Type II was heated for one hour at 60° C. and again for a second hour and was injected into mice (doses = deposit of 90 c.c.) together with (1) R colony 3 culture of Type I, (2) R colony 2 culture of Type I.

Eight mice were used for each R strain and one mouse out of each series died of Type II septicaemia in 4 and 10 days respectively. Excepting one which died prematurely and one which died in 8 days (only R colonies at the seat of inoculation) the rest were killed 16 days after inoculation and no pneumococci of any form were obtained from the subcutaneous tissues.

In another experiment the Type II S culture was heated for three hours at 60° C. and was injected in doses equivalent to 60 c.c. of broth culture. Four mice were injected with the heated cultures alone and were killed in

10 to 14 days; plate cultures from the seat of inoculation yielded no pneumococci.

The attenuated R cultures of Type I employed in this experiment were different from those used previously. They were six different colony strains from a plate from the fourth successive culture of Type I in homologous immune serum. Three mice were inoculated with 0·25 c.c. of each (total of 18 mice) together with the above mentioned heated culture. Two inoculated with different R colonies were killed, ill in 7 and 10 days respectively, and S colonies of Type II were grown from the blood. The remaining 16 were killed when well after 10 to 14 days and cultures from the subcutaneous seat of inoculation were negative except in three instances where R colonies alone were grown.

These experiments may be summarised as follows:

The injection of virulent S culture of Type II killed by heat at 60° C. together with living R strains of Type I has resulted in the formation of a virulent S culture of Type II.

The transformation has taken place when the virulent culture has been heated at 60° C. for 2 and 3 hours respectively, but the positive results were less frequent in the case of the culture heated for the longer period. Different R strains appear to vary in their ability to develop into a new S form under the influence of the heated virulent culture.

*Inoculation experiments with heated virulent culture of Types I and II together with living attenuated strains of Group IV.*

The living R strain of Group IV in the experiments in Table XIV was derived from Type II A by growth in homologous immune serum. The latter

Table XIV.

| Killed S pneumococci | Living R pneumococci | No. of mice | Result | Type of culture obtained from mice |
|---|---|---|---|---|
| Type I heated at 60° C. for 2 hours | R 1, Type II A. Dose =0·25 c.c. of blood broth culture | 5 | All died in 2–5 days | S culture of Type I from each |
| Type II heated as above | R 1, Type II A, as above | 5 | All died in 2 days | S culture of Type II from each |
| Type II A heated as above | R 1, Type II A, as above | 4 | All died in 3–5 days | S culture of Type IIA from each |
| None | R 1, Type II A. Doses =0·5–1·0 c.c. of blood broth culture | 3 | All survived | — |
| Type I heated at 60° C. for 2 hours | R 1, Pn. 41. Dose =0·2 c.c. of blood broth culture | 5 | 1 killed in 9 days<br>4 died in 4–8 days | S cultures of Pn. 41 from 4, nil from 1 |
| Type II heated at 60° C. for 2 hours | R 1, Pn. 41, as above | 5 | 2 killed in 9 days<br>3 died in 3–6 days | S cultures of Pn. 41 from 4; nil from 1 |
| Pn. 41 heated as above | R 1, Pn. 41 as above | 4 | 3 died 2–4 days<br>1 died prematurely | S cultures of Pn. 41 |
| None | R 1, Pn. 41. Dose =0·5 c.c. to 1 c.c. of culture | 3 | 1 killed in 9 days<br>2 died in 6–7 days | S culture of Pn. 41 from 2; nil from 1 |

was effective in producing attenuation, since none of five colonies selected reverted when inoculated subcutaneously in mice in doses of 10 c.c.; R colony 1 was chosen for this experiment and three control mice were inoculated.

The results show that the R strain of II A was readily transformed either into the S form of Type I or into the S form of Type II, and that reversion to its original S form occurred when it was inoculated with heated culture of that S form. The R strain of II A inoculated alone has not reverted, though larger doses than 10 c.c. have not been tried.

The second rough Group IV strain, Pn. 41, gives an interesting result and, in addition, provides a useful control for the heated cultures since, as will be seen, the heated Types I and II suspensions which were the same as those used with the rough Type II A never caused the appearance of an S strain either of Type I or Type II, thus showing that the heating had been effective in killing the S cultures.

There was no transformation of the R strain of Pn. 41 and this fact may perhaps be connected in some way with the insufficient attenuation of the strain which, as will be observed, reverts readily unaided. The R colony culture used was from one of four colonies which were picked off the plate sown from the culture in homologous serum. Evidently the serum was weak in protective substances, since two out of the four reverted on the preliminary testing. R colony 1 which failed to revert when first tested, also reverted too readily unaided when tested later.

## Inoculation of living and dead R cultures.

The experiments in Table XV are negative with one exception where an R strain derived from Type II reverted to the S form of II when inoculated into a mouse together with heated rough Type I culture.

The experiments were repeated except that six mice were used in each series = total of 24 mice. All of the mice survived.

Table XV.

| Killed R pneumococci | Living R pneumococci | No. of mice | Result | Type of culture obtained from the mouse |
|---|---|---|---|---|
| Rough Type I heated at 60° C. for 2 hours. Dose = deposit of 100 c.c. of broth culture | R 4, Type II. Dose = 0·25 c.c. of blood broth culture | 3 | Killed 16 days (2)<br>Died 4 days (1) | None<br>S colonies of Type II |
| As above | R 3, Type I. Dose = 0·25 c.c. | 4 | Killed 16 days | None |
| Rough Type II, as above | R 4, Type II. Dose = 0·25 c.c. | 4 | „ 16 „ | „ |
| As above | R 3, Type I. Dose = 0·25 c.c. | 3 | „ 16 „ | „ |

The heated R culture, although the doses were very large, viz. the deposit of 170 c.c. of broth, exerted no effect on the living R strains either in the direction of reversion or transformation of type. This is consistent with

the view that in both cases the result depends on the presence of S antigen of which there are only traces in an R strain. The heating to which the R culture had been subjected would diminish the activity of the small amount still further or destroy it entirely.

## III. Discussion.

The serological analysis of a bacterial species has an obvious practical application in bacteriological diagnosis as well as in the preparation of antibacterial therapeutic sera. There are, however, other issues, probably of greater importance, which have to do with the occurrence and remission of epidemics, the appearance of epidemic types in certain diseases and the attenuation of the infecting agent in others. It must have occurred to every serologist to ask himself the meaning of the types he has defined. Do serological types represent stages in the normal life history of a bacterium or are they the response on the part of the bacterium to changes in the immunological state of the animal host? If it is a question of altered environment, are the influences which initiated the divergence of type still at work, *i.e.* are the type characters still in a state of flux, or have the different varieties become stabilised?

On considering the above questions one cannot fail to realise that their solution would be a valuable contribution to the epidemiology of disease and would explain some of the phenomena in the rise and fall of epidemics. In certain bacterial infections, of which lobar pneumonia serves as an example, it is possible that even the most potent antisera will not avail to cut short the disease once the organisms are established in the tissues. Attention must, therefore, be directed to prevention of infection, and to this end a close study of bacterial virulence and its relation to variations in serological type is essential.

Virulence and type characters are closely related in the pneumococcus. When pneumococci are grown in homologous immune serum, some descendants become attenuated in virulence and these can be recognised by their formation on solid media of a distinctive variety of colony known as the R form of the pneumococcus. The virulent or S form of pneumococcus produces in fluid media, and still more abundantly in the peritoneal cavity of the mouse, a soluble substance which, though not itself antigenic, gives a copious precipitate with the appropriate antiserum. Each type of pneumococcus forms a special soluble substance which has no affinity for an antiserum prepared with any other pneumococcal type and it is to this property that the remarkably clear definition of the serological races of pneumococci is due. These substances have been shown[1] to consist chiefly of carbohydrate and, though highly reactive, to be non-antigenic. As a result of its change to the R form, the pneumococcus generally loses this power of producing soluble

[1] Heidelberger, M. and Avery, O. T. *J. Exp. Med.* **38**, 73 and **40**, 301.

substance, though individual strains differ in the degree of this loss. In addition to the exceptional R variety of a Group IV strain described pp. 116, 117, which produced a considerable amount and was virulent for mice, I have shown that quite attenuated R strains may form traces in the peritoneal cavity of the mouse. Cultures of R strains, however, rarely contain sufficient soluble substance to give a demonstrable precipitation with the appropriate antiserum, and, in consequence, the agglutination test no longer serves to identify a strain with the virulent type from which it was derived. Since virulence and the capacity to form soluble substances are attributes of the S strain, their possession may for convenience be ascribed to a special antigen which may be termed the S antigen. Thus an attenuated R strain which has no demonstrable S antigen has lost the serological characters of its type, but, if virulence is restored by passage through mice, the strain reverts to the S form of the type from which it was derived.

Some attenuated R strains revert readily to the virulent form and this feature is correlated with demonstrable traces of S antigen in their composition. Other strains have been found in which the R state is much more stable. In a series of peritoneal passage experiments beginning with one strain and carried on with its descendants reversion has occurred in one branch of the descent and not in another.

The acquirement of the typical characters of a virulent pneumococcus by an R strain from which the S antigen has been almost eliminated by growth in immune serum recalls some experiments by Bail on the anthrax bacillus[1]. By exposing a culture of anthrax bacilli to a temperature of 42° C. he obtained strains which were almost deprived of their power of producing capsules. Such a strain might produce a mixture of colonies some of which on subculture invariably failed to form capsules while others showed a small minority of capsule-forming bacilli. This result he ascribed to a deficient inheritance of the capsule-forming substance, so that an individual bacillus was able to endow only one of its descendants with a sufficient amount to produce a typical capsule-forming strain.

That there might be some principle underlying these infrequent and apparently haphazard positive results was suggested by the following observation. An attenuated R strain which regularly became virulent when inoculated intraperitoneally into a mouse failed to revert when the same dose was introduced into a vein. Apparently attenuated pneumococci require a protected situation in which to multiply and acquire virulence, and this they find occasionally when inoculated into the peritoneal cavity. If they are put directly into the blood stream in a dose which does not overwhelm the animal, it would appear that they do not find such suitable conditions and are readily disposed of.

This view has been confirmed by subsequent experiments and it has been found that a more certain method of ascertaining whether an R strain is capable of reverting is by the inoculation of a large dose of culture under the skin of a mouse. The mass of culture, I suppose, forms a nidus in which the

[1] *Centralbl. f. Bakt.* Orig. **79**, p. 425, 1917.

attenuated pneumococci are protected from the bactericidal action of the tissues. Since, however, attenuated pneumococci may remain alive and unaltered in the subcutaneous tissues for two or three weeks, local protection is clearly not the only factor in this method of restoring virulence. It seemed possible that the mass of R pneumococci, disintegrating under the action of the animal tissues, might furnish some substance which was utilisable by the survivors to build up their virulent structure. Acting on the assumption that this material might be the S antigenic substance, which in varying amounts persists in the R form, I inoculated into the mouse's subcutaneous tissues a very much smaller dose of living R pneumococci together with a mass of killed virulent culture. The result of this greater concentration of S antigen, or perhaps of some substance derived from it, was to make the conditions still more favourable and reversion of the R strain to the virulent form was secured with great regularity.

The observation that a sublethal dose of a bacterium may cause a fatal effect when inoculated together with the sterile products of that bacterium has long been known and forms the basis of the theory of aggressins. It was at first maintained that aggressins could only be obtained from the bacterium through contact with living animal tissues, *e.g.* from the peritoneal exudate of an animal inoculated with virulent culture, though it was finally conceded that they might be present to a slight degree in disintegrating cultures.

The action of the killed pneumococcus culture in enhancing the virulence of the R strain *in vivo* though not *in vitro* is certainly analogous to that of the hypothetical aggressin, and these results may throw fresh light on an obscure subject.

The principle of the action of aggressins was held to be their toxic influence on the leucocytes which were thus rendered incapable of attack. This is no doubt partly the function of the mass of killed virulent culture injected together with the attenuated R variety of pneumococcus, but there are other considerations which support the view already put forward that the attenuated organisms actually make use of the products of the dead culture for the synthesis of their S antigen.

An R strain is most readily transformed into the S variety when the killed culture used is of the same serological type as that from which the R strain was derived. For example, Type II S culture killed by steaming at 100° C. readily causes the R strain of Type II to revert in the mouse, whereas Type I S culture, similarly treated, does not, though it may when heated to 60° C. (*vide infra*).

An exception to the statement above is that certain Group IV strains are practically as effective as Type II in causing the R form of the latter type to revert to the S variety. The same Group IV strains, however, have no effect when injected together with an R strain of Type I. Apparently the antigens of these Group IV strains are more closely related to Type II than to Type I

and the results are further evidence of the specific selective action of different pneumococcal antigens in causing reversion.

The specific effect of the killed culture is at first sight less evident where the culture is heated to a lower temperature than 100° C. For instance, cultures of Type I heated at 60°, 70° or 75° C. frequently cause the R form derived from Type II to revert to the S form of Type II. How does this result affect the hypothesis suggested above that an attenuated R strain with deficient S substance requires the products of an S culture of the same type with which to rebuild its former type characters and virulence? One must consider first the effect of heat on the two types, I and II. Type II virulent culture, heated for so short a period as 15 minutes at 60° C., has so far never caused the R form derived from Type I to revert to the S form of Type I, although steamed cultures of Type II are effective in inducing reversion of its own R form to the corresponding S form. On the other hand, Type I while effective after heating at temperatures of 60°–75° C. in producing the R to S change with its own type loses this property when heated at 80° C. or higher.

These observations suggest that the specific S substance of Type I suffers more by exposure to heat, that is to say, a greater proportion of it is destroyed, than that of Type II.

By S substance I mean that specific protein structure of the virulent pneumococcus which enables it to manufacture a specific soluble carbohydrate. This protein seems to be necessary as material which enables the R form to build up the specific protein structure of the S form. But it appears that this material may be modified by heat in such a way that the R form cannot utilise it for the reconstruction of its own internal structure. (The specific carbohydrate which is the product of the S form is unaffected by heat.)

In order to reconcile the experimental data referred to above with the hypothesis that the R pneumococcus which reverts in the mouse to the S form has synthesised its S antigen from similar material in the heated virulent culture injected at the same time, it is necessary to assume that a virulent Type I pneumococcus contains some S antigen of Type II. An alternative hypothesis would be that the R form of Type II is able to reconstruct its virulent S form from either the S substance of Type I or that of Type II. One is then faced with the difficulty of accounting for the failure of an R form of Type I to build up its S form from Type II S substance.

The amount of S antigen of Type II in Type I must obviously be small in proportion to the Type I, since the serological tests give no indication of its presence, and it is legitimate to suppose that heating to a temperature which would not greatly diminish the total amount of Type II S antigen in a Type II pneumococcus might conceivably destroy it entirely in a Type I. This is supported by the experiments which show that after heating to 80° C. the capacity to induce reversion of the R form of Type II is lost by the Type I culture and retained by the Type II culture.

The relationship between Types I and II pneumococci just suggested, in which the major antigen of one type is represented as a subsidiary antigen in the other is not without parallel in other bacterial groups, *e.g.* meningococci. There are special circumstances, related no doubt to the formation of soluble substance, which in the ordinary serological test keep this relationship in the background.

If there is a reciprocal relationship between Types I and II, as one would expect, what is the explanation of the failure of the reversion experiments to show evidence of the presence of Type I antigen in a Type II pneumococcus? It is, I think, a question of the difference in heat resistance between the two antigens. As Type I antigen is the more heat sensitive, the small amount assumed to be present as a subsidiary antigen in a Type II pneumococcus might be destroyed by a temperature which would not affect the Type II antigen in a Type I pneumococcus.

These considerations, I think, afford a reasonable explanation of the experimental data in connection with the restoration of virulence to an attenuated pneumococcus. The chief points are:—(1) in the change from the S to the R form some of the S antigen may persist; the amount, rarely demonstrable by *in vitro* tests, varies in different R strains; (2) the S antigen remaining in an R strain may be regenerated and reach its original abundance under suitable conditions, *e.g.* inoculation subcutaneously into a mouse in large doses or in small doses plus a mass of heated culture containing the particular S antigen; (3) an S strain of one type (I or II) may contain in addition to its major antigen a remnant of the other type antigen.

Application of the principles underlying these observations to the question of transformation of one type into another has given results of considerable interest.

When pneumococci of Types I and II are reduced to their respective R forms by growth in homologous immune sera, they lose nearly all their major S antigen though they may retain their minor S antigens which are presumably not affected by the heterologous immune substances. But the major S antigen apparently still preponderates, since an R strain on reversion to the S form regains its original type characters. Some R strains, however, do not revert even when inoculated in large amounts under the skin of a mouse, and it is not unlikely that in such strains the major antigen has been reduced to the same insignificant amount as the minor antigen.

In such circumstances an R strain derived from Type I would be identical with an R strain from Type II, and under suitable conditions the development from it of a virulent form of either type might be anticipated.

This has been shown actually to occur; a virulent Type I pneumococcus can be derived through the intermediary R form from a virulent Type II pneumococcus, and *vice versa*.

Up to the present I have maintained a distinction between the R forms derived from Types I and II respectively, though, as I have stated earlier,

they can be identified only if they revert unaided to the S type from which they originated.

When the R form of either type is furnished under suitable experimental conditions with a mass of the S form of the other type, it appears to utilise that antigen as a pabulum from which to build up a similar antigen and thus to develop into an S strain of that type. Therefore the R form of Type II, when inoculated together with a heated suspension of Type I, uses the antigen of the latter strain and an S pneumococcus of Type I makes its appearance. There is a further complication since, as it appears, the heated Type I suspension contains a subsidiary S antigen of Type II, and some of the R pneumococci may use this to develop an S strain of Type II. As a result one mouse may yield the S form of Type I and another the S form of Type II, while quite frequently the same mouse may yield both types.

Similarly, if the R form of Type I is inoculated together with the heated S culture of Type II, a virulent S form of Type II is developed. (The S culture of Type II never causes the R form of Type I to change into the corresponding S form, because, as I have already explained, the subsidiary Type I antigen is destroyed by the heating.)

These observations suggest that there is no essential distinction between the two R varieties. In fact, there are certain indications that the R pneumococcus in its ultimate form is the same, no matter from what type it is derived; it possesses both Types I and II antigens in a rudimentary form or, as it may be differently expressed, it is able to develop either S form according to the material available.

If Type III substance is offered as a pabulum, either form is able to build up a typical S strain of Type III, though it appears that the R form derived from Type II is more readily converted into Type III than is the R form of Type I. Why there should be this difference is not clear, though it may be assumed to have some association with the fact that this particular R strain from Type II has not reached the same stage of attenuation as the R strain of Type I. (The latter in the relatively few tests made has not reverted, as does the R Type II, to the S form when inoculated unaided, *i.e.* alone in large doses.) There is also a further point of distinction in that injection of heated Group IV cultures has caused the R form of Type II to revert but not the R form of Type I. The S substance of the Group IV strains is evidently closely related to that of Type II since it provides a suitable pabulum for the regeneration of the virulent S strain. In this connection I may recall that Group IV strains appear in the sputum during convalescence from Type I pneumonia, and it is suggested that they are formed from the Type I after suppression of the major antigen through the action of the immune substances and by the development of the subsidiary Type II antigen, though not to its full complexity.

It may be that the minor antigen in a Type I is not actually a fully developed Type II S antigen in small amount but a less differentiated substance

which serves indifferently as a foundation for the building up of either Type II or a Group IV strain.

The R form derived from a Group IV strain, viz. Type II A, can be transformed into the S forms of Type I or Type II or changed back to its original S form according to the particular S substance which is injected along with it. On the other hand, another Group IV strain which was incompletely attenuated invariably reverted to its original S form no matter what type of S culture was injected with it. It seems that, if a pneumococcus has a moderately well-developed S structure, there is no tendency to develop into an S variety of any other type.

The method by which transformation of type has been secured consists in heating to 60° C. for 15 minutes up to 3 hours a virulent culture of one type and inoculating a large amount of the heated culture under the skin of a mouse together with a small dose of the R strain derived from another type.

Experiments with culture heated at temperatures higher than 60° C. have rarely been successful in causing transformation of type. In one instance the S form of Type I was obtained from a mouse which had been inoculated with the R form derived from Type II together with a suspension of Type I heated to 70° C. for 15 minutes, in a second the S form of Type II was obtained from a mouse inoculated with the R form of Type I together with a virulent Type II culture heated to 65° C. for 15 minutes.

The question arises whether heating at the above temperatures had in fact killed all the individual pneumococci in the mass of virulent culture or whether the apparent change of type was due to the occurrence of a survivor. I have given this question careful consideration and I have never been able by the ordinary methods of culture and animal inoculation to demonstrate the presence of viable organisms in the heated cultures. Since there is no reason to suppose, and I have had no evidence to show, that the R strains used were mixed, there seems to be no alternative to the hypothesis of transformation of type.

A few years ago the statement that a Type I strain could be changed into a Type II or a Type III would have been received with greater scepticism than at the present day. Since, however, it has been shown that a pneumococcus can readily be deprived of its type characters and virulence, and that under favourable conditions these can be restored, the possibility appears less unlikely.

The apparent transformation is not an abrupt change of one type into another, but a process of evolution through an intermediate stage, the R form, from which the type characters have been obliterated. Mutation of type among disease-producing bacteria is a subject of obvious importance in the study of epidemiological problems. If it can be proved to occur in the pneumococcus group with its sharply defined immunological races, the possibility can hardly be denied to other bacterial groups where the serological

types cannot be differentiated without the help of agglutinin-absorption experiments.

The position in regard to the causal relation of different types of pneumococci to lobar pneumonia presents certain difficulties. Types I and II pneumococci, which cause 60 to 70 per cent. of the total cases of lobar pneumonia, are rarely found in the normal nasopharynx except in close contacts of the disease. Whilst this latter observation indicates some power of epidemic spread, it is not often demonstrable that a case of pneumonia acts as a focus for fresh cases. On the other hand, Group IV pneumococci are of common occurrence in the nasopharynx and about 25 to 30 per cent. of pneumonia cases are attributable to various types in this group; these cases are generally considered to be of autogenous origin.

It is a very remarkable fact that the incidence of the chief types of pneumococci in lobar pneumonia is almost identical in countries where the climatic and social conditions are similar. While this occurrence is not easily explained on the supposition that the disease is partly infectious and partly autogenous, it is not inconsistent with the evolution in the individual of special types most suited to set up pneumonia, *i.e.* the similar distribution is due to similar composition of the population as regards susceptibility to the pneumococcus and not to similarity in the diffusion of pneumococcal types.

In convalescence from pneumonia Types I and II tend to disappear from the respiratory tract and are replaced by the common Group IV strains. According to the more generally accepted view, the chief types die out and the Group IV pneumococci, the normal inhabitants of the nasopharynx, again come into prominence. An alternative hypothesis which was purely speculative in the absence of evidence of the instability of pneumococcal types is that the chief types revert to the Group IV varieties from which they were derived during the development of the disease in the individual.

On the lines of my previous argument as to the process at work in the development of a virulent S strain from an attenuated R pneumococcus, it may be surmised that the immune substances formed during recovery suppress the S antigens of the chief type and under suitable conditions the subsidiary antigens are developed to form a new virulent type—in this case one of the varieties of Group IV. As mentioned earlier, I have shown that the sputum of a case of pneumonia due to Type I almost invariably contains, in addition, one or more virulent strains of Group IV, and as many as four distinct types have been isolated from a single case.

This latter instance certainly suggests that the Group IV strains are variants of the Type I, and it is of some significance in this connection that the antigens of the Group IV strains have been shown to be related to that of Type II which is represented as a subsidiary antigen in a Type I pneumococcus. It would appear that the Type I antigen no longer serves its purpose in the presence of the immune substances formed during convalescence, and

the pneumococcus consequently develops its Type II side. I have not so far been able to find a Group IV strain in a case of pneumonia due to Type II (in one instance a Type III strain was found in association with the Type II), but my observations have not been sufficiently numerous to justify a conclusion on this point.

While these suggestions of a regular sequence of changes in the type of pneumococcus before the development of pneumonia and during recovery are necessarily tentative in character, they are in harmony with the experimental data and amplify this line of thought.

The formation of a Group IV strain from a Type I might be considered as an adaptation on the part of the Type I pneumococcus to the altered conditions consequent on the development of immune bodies. These make it difficult for the Type I to survive as such (*in vitro* the R form is the response), and in assuming Group IV characters it makes some sacrifice of its antigenic complexity and, with that, of infectivity in exchange for a greater degree of resisting power to the animal tissues. Type III exhibits a still greater instance of change in that direction, since it is very slightly invasive but fatal in its effects once it is established in the body. It is more difficult to produce protective sera in rabbits with Group IV and Type III strains than with Type I, while Type II occupies an intermediate position.

In the interaction between the animal tissues and the bacterium one is apt to consider the bacterium as playing a purely passive part and to overlook the possibility that the various forms and types may be assumed by it to meet alterations in its environment.

What, for instance, is the meaning of the change to the R form? Most writers have regarded it as a degenerative change due to unfavourable conditions for growth. While this is true in a sense, since in the R form the bacterium lacks certain important attributes characteristic of the S form, there is some evidence of its being rather a vital adaptation, as P. Hadley[1] has suggested.

In the case of the pneumococcus the change to the R form is brought about most rapidly in immune serum which, nevertheless, provides an excellent medium for the growth of both the R and the S form. The effect of the serum is due to the specific immune substances, and, as a result, the pneumococcus becomes susceptible to phagocytic action. If, as is probable, the immune bodies exercise a similar influence *in vivo* during successful resistance, the animal has achieved its end in rendering the invader harmless. By assuming the R form the pneumococcus has admitted defeat, but has made such efforts as are possible to retain the potentiality to develop afresh into a virulent organism. The immune substances do not apparently continue to act on the pneumococcus after it has reached the R stage, and it is thus able to preserve remnants of its important S antigens and with them the capacity to revert to the virulent form.

[1] *The Journal of Infectious Diseases*, 1927, **40**, pp. 1–312.

While the R form may be the final stage in the struggle of the bacterium to preserve its individuality, I look upon the occurrence of the various serological races as evidence of similar efforts to contend against adverse circumstances. These are more successful in that the S form is retained and, in addition, increased powers of resistance are acquired but at the sacrifice of invasive properties.

The experiments on enhancement of virulence and transformation of type suggest an explanation of the manner in which a pneumococcus residing as an apparently harmless saprophyte in the nasopharynx acquires disease-producing powers. So long as it retains certain potentialities, indicated by the possession of traces of S antigen, the most attenuated pneumococcus may develop the full equipment of virulence. The first essential is a situation in which it can multiply, unchecked by the inhibitory action of a healthy mucous membrane. In the nidus thus formed the pneumococcus gradually builds up from material furnished by its disintegrating companions an antigenic structure with invasive properties sufficient to cope with the resistance of its host.

When recovery from pneumonia takes place, the formation of immune substances initiates the retrogressive changes in antigen structure resulting in the production of the Group IV and Type III pneumococci which probably have increased resisting powers but diminished capacity for invasion.

These considerations which relate to an individual case of pneumonia are capable of application to an outbreak of epidemic disease in a community. Thus the consequences which ensue on the decline of an epidemic are not only an increase in the number of insusceptible individuals but also an alteration in the character of the infective organism.

## IV. SUMMARY.

1. In the course of the examination of sputum from cases of lobar pneumonia, observations have been made on the incidence of the chief types of pneumococci. In the district from which the material was obtained, there was an apparent local diminution in the number of cases of lobar pneumonia due to Type II; the figures were 32·6 per cent. of Type II cases in the period 1920–22, and only 7·4 per cent. in the period 1924–27. The incidence of Type I was approximately the same in the two periods, the percentages being 30·6 and 34·3.

2. Several different serological varieties of pneumococci have been obtained from the sputum of each of several cases of pneumonia examined at various stages of the disease. This has occurred most frequently in cases of pneumonia due to Type I, and in two instances four different types of Group IV were found in addition to the chief types. The recovery of different types is facilitated by the inoculation of the sputum (preserved in the refrigerator), together with protective sera corresponding to the various types in the order of their appearance.

3. Two interesting strains of Group IV pneumococci have been obtained from pneumonic sputum.

One was an R strain which produced typical rough colonies, yet preserved its virulence for mice and its capacity to form soluble substance. This R pneumococcus developed a large capsule in the mice, which died of a chronic type of septicaemia. A strain producing smooth colonies was obtained from it in the course of a prolonged series of passage experiments.

The second strain, which was proved not to be a mixture, agglutinated specifically with the sera of two different types. In the peritoneal cavity of the mouse the specific soluble substance of each type was produced.

4. A method of producing the S to R change through ageing of colonies on chocolate blood medium containing horse serum is described. After two to three days' incubation small rough patches appear in the margins of the smooth colonies, and from these pure R strains can be isolated.

5. It has been shown that the R change is not equally advanced in the descendants of virulent pneumococci which have been exposed to the action of homologous immune serum. Some R strains form traces of soluble substance in the peritoneal cavity of the mouse; these revert readily to the virulent S form and, in addition, are able to produce active immunity. Others show no evidence of S antigen; spontaneous reversion takes place with difficulty, if at all, and they are incapable of producing active immunity. The stronger the immune serum used, the more permanent and complete is the change to the R form.

6. Restoration of virulence to an attenuated R strain, with recovery of the S form of colony and of the original serological type characters may be obtained by passage through mice. The change from the R to the S form is favoured by the inoculation of the R culture in large doses into the subcutaneous tissues; but the most certain method of procuring reversion is by the inoculation of the R culture, subcutaneously into a mouse, together with a large dose of virulent culture of the same type killed by heat.

Incubation of such a mixture *in vitro* does not induce reversion.

7. Reversion of an R strain to its S form may occasionally be brought about by the simultaneous inoculation of virulent culture of another type, especially when this has been heated for only a short period to 60° C., *e.g.* R Type II to its S form when inoculated with heated Type I culture.

8. Type I antigen appears to be more sensitive to exposure to heat than Type II antigen, since the former loses the power to cause reversion when heated to 80° C., whereas Type II culture remains effective even after steaming at 100° C.

9. The antigens of certain Group IV strains appear to be closely related to that of Type II, and are equally resistant to heat. Steamed cultures of these Group IV strains cause the R form derived from Type II to revert to its S form, while they fail to produce reversion of the R form derived from Type I.

10. The inoculation into the subcutaneous tissues of mice of an attenuated R strain derived from one type, together with a large dose of virulent culture of another type killed by heating to 60° C., has resulted in the formation of a virulent S pneumococcus of the same type as that of the heated culture.

The newly formed S strain may remain localised at the seat of inoculation, or it may disseminate and cause fatal septicaemia.

The S form of Type I has been produced from the R form of Type II, and the R form of Type I has been transformed into the S form of Type II.

The clear mucinous colonies of Type III have been derived both from the R form of Type I and from the R form of Type II, though they appear to be produced more readily from the latter. The newly formed strains of Type III have been of relatively low virulence, and have frequently remained localised at the subcutaneous seat of inoculation.

Virulent strains of Types I and II have been obtained from an R strain of Group IV.

11. Heated R cultures injected in large doses, together with small doses of living R culture have never caused transformation of type, and only rarely produced a reversion of the R form of Type II to its virulent S form.

12. The results of the experiments on enhancement of virulence and on transformation of type are discussed and their significance in regard to questions of epidemiology is indicated.

(*MS. received for publication* 26. VIII. 1927.—Ed.)

# STUDIES ON THE CHEMICAL NATURE OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES

## Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III

By OSWALD T. AVERY, M.D., COLIN M. MacLEOD, M.D., and MACLYN McCARTY,* M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

Plate 1

(Received for publication, November 1, 1943)

Biologists have long attempted by chemical means to induce in higher organisms predictable and specific changes which thereafter could be transmitted in series as hereditary characters. Among microörganisms the most striking example of inheritable and specific alterations in cell structure and function that can be experimentally induced and are reproducible under well defined and adequately controlled conditions is the transformation of specific types of Pneumococcus. This phenomenon was first described by Griffith (1) who succeeded in transforming an attenuated and non-encapsulated (R) variant derived from one specific type into fully encapsulated and virulent (S) cells of a heterologous specific type. A typical instance will suffice to illustrate the techniques originally used and serve to indicate the wide variety of transformations that are possible within the limits of this bacterial species.

Griffith found that mice injected subcutaneously with a small amount of a living R culture derived from Pneumococcus Type II together with a large inoculum of heat-killed Type III (S) cells frequently succumbed to infection, and that the heart's blood of these animals yielded Type III pneumococci in pure culture. The fact that the R strain was avirulent and incapable by itself of causing fatal bacteremia and the additional fact that the heated suspension of Type III cells contained no viable organisms brought convincing evidence that the R forms growing under these conditions had newly acquired the capsular structure and biological specificity of Type III pneumococci.

The original observations of Griffith were later confirmed by Neufeld and Levinthal (2), and by Baurhenn (3) abroad, and by Dawson (4) in this laboratory. Subsequently Dawson and Sia (5) succeeded in inducing transformation *in vitro*. This they accomplished by growing R cells in a fluid medium containing anti-R serum and heat-killed encapsulated S cells. They showed that in the test tube as in the animal body transformation can be selectively induced, depending on the type specificity of the S cells used in the reaction system. Later, Alloway (6) was able to cause

---

* Work done in part as Fellow in the Medical Sciences of the National Research Council.

specific transformation *in vitro* using sterile extracts of S cells from which all formed elements and cellular debris had been removed by Berkefeld filtration. He thus showed that crude extracts containing active transforming material in soluble form are as effective in inducing specific transformation as are the intact cells from which the extracts were prepared.

Another example of transformation which is analogous to the interconvertibility of pneumococcal types lies in the field of viruses. Berry and Dedrick (7) succeeded in changing the virus of rabbit fibroma (Shope) into that of infectious myxoma (Sanarelli). These investigators inoculated rabbits with a mixture of active fibroma virus together with a suspension of heat-inactivated myxoma virus and produced in the animals the symptoms and pathological lesions characteristic of infectious myxomatosis. On subsequent animal passage the transformed virus was transmissible and induced myxomatous infection typical of the naturally occurring disease. Later Berry (8) was successful in inducing the same transformation using a heat-inactivated suspension of washed elementary bodies of myxoma virus. In the case of these viruses the methods employed were similar in principle to those used by Griffith in the transformation of pneumococcal types. These observations have subsequently been confirmed by other investigators (9).

The present paper is concerned with a more detailed analysis of the phenomenon of transformation of specific types of Pneumococcus. The major interest has centered in attempts to isolate the active principle from crude bacterial extracts and to identify if possible its chemical nature or at least to characterize it sufficiently to place it in a general group of known chemical substances. For purposes of study, the typical example of transformation chosen as a working model was the one with which we have had most experience and which consequently seemed best suited for analysis. This particular example represents the transformation of a non-encapsulated R variant of Pneumococcus Type II to Pneumococcus Type III.

## EXPERIMENTAL

Transformation of pneumococcal types *in vitro* requires that certain cultural conditions be fulfilled before it is possible to demonstrate the reaction even in the presence of a potent extract. Not only must the broth medium be optimal for growth but it must be supplemented by the addition of serum or serous fluid known to possess certain special properties. Moreover, the R variant, as will be shown later, must be in the reactive phase in which it has the capacity to respond to the transforming stimulus. For purposes of convenience these several components as combined in the transforming test will be referred to as the *reaction system.* Each constituent of this system presented problems which required clarification before it was possible to obtain consistent and reproducible results. The various components of the system will be described in the following order: (1) nutrient broth, (2) serum or serous fluid, (3) strain of R Pneumococcus, and (4) extraction, purification, and chemical nature of the transforming principle.

*1. Nutrient Broth.*—Beef heart infusion broth containing 1 per cent neopeptone with no added dextrose and adjusted to an initial pH of 7.6–7.8 is used as the basic medium. Individual lots of broth show marked and unpredictable variations in the property of supporting transformation. It has been found, however, that charcoal adsorption, according to the method described by MacLeod and Mirick (10) for removal of sulfonamide inhibitors, eliminates to a large extent these variations; consequently this procedure is used as routine in the preparation of consistently effective broth for titrating the transforming activity of extracts.

*2. Serum or Serous Fluid.*—In the first successful experiments on the induction of transformation *in vitro*, Dawson and Sia (5) found that it was essential to add serum to the medium. Anti-R pneumococcal rabbit serum was used because of the observation that reversion of an R pneumococcus to the homologous S form can be induced by growth in a medium containing anti-R serum. Alloway (6) later found that ascitic or chest fluid and normal swine serum, all of which contain R antibodies, are capable of replacing antipneumococcal rabbit serum in the reaction system. Some form of serum is essential, and to our knowledge transformation *in vitro* has never been effected in the absence of serum or serous fluid.

In the present study human pleural or ascitic fluid has been used almost exclusively. It became apparent, however, that the effectiveness of different lots of serum varied and that the differences observed were not necessarily dependent upon the content of R antibodies, since many sera of high titer were found to be incapable of supporting transformation. This fact suggested that factors other than R antibodies are involved.

It has been found that sera from various animal species, irrespective of their immune properties, contain an enzyme capable of destroying the transforming principle in potent extracts. The nature of this enzyme and the specific substrate on which it acts will be referred to later in this paper. This enzyme is inactivated by heating the serum at 60°–65°C., and sera heated at temperatures known to destroy the enzyme are often rendered effective in the transforming system. Further analysis has shown that certain sera in which R antibodies are present and in which the enzyme has been inactivated may nevertheless fail to support transformation. This fact suggests that still another factor in the serum is essential. The content of this factor varies in different sera, and at present its identity is unknown.

There are at present no criteria which can be used as a guide in the selection of suitable sera or serous fluids except that of actually testing their capacity to support transformation. Fortunately, the requisite properties are stable and remain unimpaired over long periods of time; and sera that have been stored in the refrigerator for many months have been found on retesting to have lost little or none of their original effectiveness in supporting transformation.

The recognition of these various factors in serum and their rôle in the reaction system has greatly facilitated the standardization of the cultural conditions required for obtaining consistent and reproducible results.

*3. The R Strain (R36A).*—The unencapsulated R strain used in the present study was derived from a virulent "S" culture of Pneumococcus Type II. It will be recalled that irrespective of type derivation all "R" variants of Pneumococcus are characterized by the lack of capsule formation and the

consequent loss of both type specificity and the capacity to produce infection in the animal body. The designation of these variants as R forms has been used to refer merely to the fact that on artificial media the colony surface is "rough" in contrast to the smooth, glistening surface of colonies of encapsulated S cells.

The R strain referred to above as R36A was derived by growing the parent S culture of Pneumococcus Type II in broth containing Type II antipneumococcus rabbit serum for 36 serial passages and isolating the variant thus induced. The strain R36A has lost all the specific and distinguishing characteristics of the parent S organisms and consists only of attenuated and non-encapsulated R variants. The change S → R is often a reversible one provided the R cells are not too far "degraded." The reversion of the R form to its original specific type can frequently be accomplished by successive animal passages or by repeated serial subculture in anti-R serum. When reversion occurs under these conditions, however, the R culture invariably reverts to the encapsulated form of the same specific type as that from which it was derived (11). Strain R36A has become relatively fixed in the R phase and has never spontaneously reverted to the Type II S form. Moreover, repeated attempts to cause it to revert under the conditions just mentioned have in all instances been unsuccessful.

The reversible conversion of S⇌R within the limits of a single type is quite different from the transformation of one specific type of Pneumococcus into another specific type through the R form. Transformation of types has never been observed to occur spontaneously and has been induced experimentally only by the special techniques outlined earlier in this paper. Under these conditions, the enzymatic synthesis of a chemically and immunologically different capsular polysaccharide is specifically oriented and selectively determined by the specific type of S cells used as source of the transforming agent.

In the course of the present study it was noted that the stock culture of R36 on serial transfers in blood broth undergoes spontaneous dissociation giving rise to a number of other R variants which can be distinguished one from another by colony form. The significance of this in the present instance lies in the fact that of four different variants isolated from the parent R culture only one (R36A) is susceptible to the transforming action of potent extracts, while the others fail to respond and are wholly inactive in this regard. The fact that differences exist in the responsiveness of different R variants to the same specific stimulus enphasizes the care that must be exercised in the selection of a suitable R variant for use in experiments on transformation. The capacity of this R strain (R36A) to respond to a variety of different transforming agents is shown by the readiness with which it can be transformed to Types I, III, VI, or XIV, as well as to its original type (Type II), to which, as pointed out, it has never spontaneously reverted.

Although the significance of the following fact will become apparent later on, it must be mentioned here that pneumococcal cells possess an enzyme capable of destroying the activity of the transforming principle. Indeed, this enzyme has been

found to be present and highly active in the autolysates of a number of different strains. The fact that this intracellular enzyme is released during autolysis may explain, in part at least, the observation of Dawson and Sia (5) that it is essential in bringing about transformation in the test tube to use a small inoculum of young and actively growing R cells. The irregularity of the results and often the failure to induce transformation when large inocula are used may be attributable to the release from autolyzing cells of an amount of this enzyme sufficient to destroy the transforming principle in the reaction system.

In order to obtain consistent and reproducible results, two facts must be borne in mind: first, that an R culture can undergo spontaneous dissociation and give rise to other variants which have lost the capacity to respond to the transforming stimulus; and secondly, that pneumococcal cells contain an intracellular enzyme which when released destroys the activity of the transforming principle. Consequently, it is important to select a responsive strain and to prevent as far as possible the destructive changes associated with autolysis.

*Method of Titration of Transforming Activity.*—In the isolation and purification of the active principle from crude extracts of pneumococcal cells it is desirable to have a method for determining quantitatively the transforming activity of various fractions.

The experimental procedure used is as follows: Sterilization of the material to be tested for activity is accomplished by the use of alcohol since it has been found that this reagent has no effect on activity. A measured volume of extract is precipitated in a sterile centrifuge tube by the addition of 4 to 5 volumes of absolute ethyl alcohol, and the mixture is allowed to stand 8 or more hours in the refrigerator in order to effect sterilization. The alcohol precipitated material is centrifuged, the supernatant discarded, and the tube containing the precipitate is allowed to drain for a few minutes in the inverted position to remove excess alcohol. The mouth of the tube is then carefully flamed and a dry, sterile cotton plug is inserted. The precipitate is redissolved in the original volume of saline. Sterilization of active material by this technique has invariably proved effective. This procedure avoids the loss of active substance which may occur when the solution is passed through a Berkefeld filter or is heated at the high temperatures required for sterilization.

To the charcoal-adsorbed broth described above is added 10 per cent of the sterile ascitic or pleural fluid which has previously been heated at 60°C. for 30 minutes, in order to destroy the enzyme known to inactivate the transforming principle. The enriched medium is distributed under aseptic conditions in 2.0 cc. amounts in sterile tubes measuring 15 × 100 mm. The sterilized extract is diluted serially in saline neutralized to pH 7.2–7.6 by addition of 0.1 N NaOH, or it may be similarly diluted in M/40 phosphate buffer, pH 7.4. 0.2 cc. of each dilution is added to at least 3 or 4 tubes of the serum medium. The tubes are then seeded with a 5 to 8 hour blood broth culture of R36A. 0.05 cc. of a $10^{-4}$ dilution of this culture is added to each tube, and the cultures are incubated at 37°C. for 18 to 24 hours.

The anti-R properties of the serum in the medium cause the R cells to agglutinate during growth, and clumps of the agglutinated cells settle to the bottom of the tube leaving a clear supernatant. When transformation occurs, the encapsulated S cells, not being affected by these antibodies, grow diffusely throughout the medium. On the other hand, in the absence of transformation the supernatant remains clear, and only sedimented growth of R organisms occurs. This difference in the character of growth makes it possible by inspection alone to distinguish tentatively between positive and negative results. As routine all the cultures are plated on blood agar for confirmation and further bacteriological identification. Since the extracts used in the present study were derived from Pneumococcus Type III, the differentiation between the colonies of the original R organism and those of the transformed S cells is especially striking, the latter being large, glistening, mucoid colonies typical of Pneumococcus Type III. Figs. 1 and 2 illustrate these differences in colony form.

A typical protocol of a titration of the transforming activity of a highly purified preparation is given in Table IV.

### *Preparative Methods*

*Source Material.*—In the present investigation a stock laboratory strain of Pneumococcus Type III (A66) has been used as source material for obtaining the active principle. Mass cultures of these organisms are grown in 50 to 75 liter lots of plain beef heart infusion broth. After 16 to 18 hours' incubation at 37°C. the bacterial cells are collected in a steam-driven sterilizable Sharples centrifuge. The centrifuge is equipped with cooling coils immersed in ice water so that the culture fluid is thoroughly chilled before flowing into the machine. This procedure retards autolysis during the course of centrifugation. The sedimented bacteria are removed from the collecting cylinder and resuspended in approximately 150 cc. of chilled saline (0.85 per cent NaCl), and care is taken that all clumps are thoroughly emulsified. The glass vessel containing the thick, creamy suspension of cells is immersed in a water bath, and the temperature of the suspension rapidly raised to 65°C. During the heating process the material is constantly stirred, and the temperature maintained at 65°C. for 30 minutes. Heating at this temperature inactivates the intracellular enzyme known to destroy the transforming principle.

*Extraction of Heat-Killed Cells.*—Although various procedures have been used, only that which has been found most satisfactory will be described here. The heat-killed cells are washed with saline 3 times. The chief value of the washing process is to remove a large excess of capsular polysaccharide together with much of the protein, ribonucleic acid, and somatic "C" polysaccharide. Quantitative titrations of transforming activity have shown that not more than 10 to 15 per cent of the active material is lost in the washing, a loss which is small in comparison to the amount of inert substances which are removed by this procedure.

After the final washing, the cells are extracted in 150 cc. of saline containing sodium desoxycholate in final concentration of 0.5 per cent by shaking the mixture me-

chanically 30 to 60 minutes. The cells are separated by centrifugation, and the extraction process is repeated 2 or 3 times. The desoxycholate extracts prepared in this manner are clear and colorless. These extracts are combined and precipitated by the addition of 3 to 4 volumes of absolute ethyl alcohol. The sodium desoxycholate being soluble in alcohol remains in the supernatant and is thus removed at this step. The precipitate forms a fibrous mass which floats to the surface of the alcohol and can be removed directly by lifting it out with a spatula. The excess alcohol is drained from the precipitate which is then redissolved in about 50 cc. of saline. The solution obtained is usually viscous, opalescent, and somewhat cloudy.

*Deproteinization and Removal of Capsular Polysaccharide.*—The solution is then deproteinized by the chloroform method described by Sevag (12). The procedure is repeated 2 or 3 times until the solution becomes clear. After this preliminary treatment the material is reprecipitated in 3 to 4 volumes of alcohol. The precipitate obtained is dissolved in a larger volume of saline (150 cc.) to which is added 3 to 5 mg. of a purified preparation of the bacterial enzyme capable of hydrolyzing the Type III capsular polysaccharide (13). The mixture is incubated at 37°C., and the destruction of the capsular polysaccharide is determined by serological tests with Type III antibody solution prepared by dissociation of immune precipitate according to the method described by Liu and Wu (14). The advantages of using the antibody solution for this purpose are that it does not react with other serologically active substances in the extract and that it selectively detects the presence of the capsular polysaccharide in dilutions as high as 1:6,000,000. The enzymatic breakdown of the polysaccharide is usually complete within 4 to 6 hours, as evidenced by the loss of serological reactivity. The digest is then precipitated in 3 to 4 volumes of ethyl alcohol, and the precipitate is redissolved in 50 cc. of saline. Deproteinization by the chloroform process is again used to remove the added enzyme protein and remaining traces of pneumococcal protein. The procedure is repeated until no further film of protein-chloroform gel is visible at the interface.

*Alcohol Fractionation.*—Following deproteinization and enzymatic digestion of the capsular polysaccharide, the material is repeatedly fractionated in ethyl alcohol as follows. Absolute ethyl alcohol is added dropwise to the solution with constant stirring. At a critical concentration varying from 0.8 to 1.0 volume of alcohol the active material separates out in the form of fibrous strands that wind themselves around the stirring rod. This precipitate is removed on the rod and washed in a 50 per cent mixture of alcohol and saline. Although the bulk of active material is removed by fractionation at the critical concentration, a small but appreciable amount remains in solution. However, upon increasing the concentration of alcohol to 3 volumes, the residual fraction is thrown down together with inert material in the form of a flocculent precipitate. This flocculent precipitate is taken up in a small volume of saline (5 to 10 cc.) and the solution again fractionated by the addition of 0.8 to 1.0 volume of alcohol. Additional fibrous material is obtained which is combined with that recovered from the original solution. Alcoholic fractionation is repeated 4 to 5 times. The yield of fibrous material obtained by this method varies from 10 to 25 mg. per 75 liters of culture and represents the major portion of active material present in the original crude extract.

*Effect of Temperature.*—As a routine procedure all steps in purification were carried

out at room temperature unless specifically stated otherwise. Because of the theoretical advantage of working at low temperature in the preparation of biologically active material, the purification of one lot (preparation 44) was carried out in the cold. In this instance all the above procedures with the exception of desoxycholate ex-extraction and enzyme treatment were conducted in a cold room maintained at 0–4°C. This preparation proved to have significantly higher activity than did material similarly prepared at room temperature.

Desoxycholate extraction of the heat-killed cells at low temperature is less efficient and yields smaller amounts of the active fraction. It has been demonstrated that higher temperatures facilitate extraction of the active principle, although activity is best preserved at low temperatures.

### *Analysis of Purified Transforming Material*

*General Properties.*—Saline solutions containing 0.5 to 1.0 mg. per cc. of the purified substance are colorless and clear in diffuse light. However, in strong transmitted light the solution is not entirely clear and when stirred exhibits a silky sheen. Solutions at these concentrations are highly viscous.

Purified material dissolved in physiological salt solution and stored at 2–4°C. retains its activity in undiminished titer for at least 3 months. However, when dissolved in distilled water, it rapidly decreases in activity and becomes completely inert within a few days. Saline solutions stored in the frozen state in a $CO_2$ ice box (−70°C.) retain full potency for several months. Similarly, material precipitated from saline solution by alcohol and stored under the supernatant remains active over a long period of time. Partially purified material can be preserved by drying from the frozen state in the lyophile apparatus. However, when the same procedure is used for the preservation of the highly purified substance, it is found that the material undergoes changes resulting in decrease in solubility and loss of activity.

The activity of the transforming principle in crude extracts withstands heating for 30 to 60 minutes at 65°C. Highly purified preparations of active material are less stable, and some loss of activity occurs at this temperature. A quantitative study of the effect of heating purified material at higher temperatures has not as yet been made. Alloway (6), using crude extracts prepared from Type III pneumococcal cells, found that occasionally activity could still be demonstrated after 10 minutes' exposure in the water bath to temperatures as high as 90°C.

The procedures mentioned above were carried out with solutions adjusted to neutral reaction, since it has been shown that hydrogen ion concentrations in the acid range result in progressive loss of activity. Inactivation occurs rapidly at pH 5 and below.

*Qualitative Chemical Tests.*—The purified material in concentrated solution gives negative biuret and Millon tests. These tests have been done directly on dry material with negative results. The Dische diphenylamine reaction

for desoxyribonucleic acid is strongly positive. The orcinol test (Bial) for ribonucleic acid is weakly positive. However, it has been found that in similar concentrations pure preparations of desoxyribonucleic acid of animal origin prepared by different methods give a Bial reaction of corresponding intensity.

Although no specific tests for the presence of lipid in the purified material have been made, it has been found that crude material can be repeatedly extracted with alcohol and ether at −12°C. without loss of activity. In addition, as will be noted in the preparative procedures, repeated alcohol precipitation and treatment with chloroform result in no decrease in biological activity.

*Elementary Chemical Analysis.*[1]—Four purified preparations were analyzed for content of nitrogen, phosphorus, carbon, and hydrogen. The results are presented in Table I. The nitrogen-phosphorus ratios vary from 1.58 to 1.75 with an average value of 1.67 which is in close agreement with that calculated

TABLE I

*Elementary Chemical Analysis of Purified Preparations of the Transforming Substance*

| Preparation No. | Carbon | Hydrogen | Nitrogen | Phosphorus | N/P ratio |
|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | |
| 37 | 34.27 | 3.89 | 14.21 | 8.57 | 1.66 |
| 38B | — | — | 15.93 | 9.09 | 1.75 |
| 42 | 35.50 | 3.76 | 15.36 | 9.04 | 1.69 |
| 44 | — | — | 13.40 | 8.45 | 1.58 |
| Theory for sodium desoxyribonucleate..... | 34.20 | 3.21 | 15.32 | 9.05 | 1.69 |

on the basis of the theoretical structure of sodium desoxyribonucleate (tetranucleotide). The analytical figures by themselves do not establish that the substance isolated is a pure chemical entity. However, on the basis of the nitrogen-phosphorus ratio, it would appear that little protein or other substances containing nitrogen or phosphorus are present as impurities since if they were this ratio would be considerably altered.

*Enzymatic Analysis.*—Various crude and crystalline enzymes[2] have been tested for their capacity to destroy the biological activity of potent bacterial extracts. Extracts buffered at the optimal pH, to which were added crystalline trypsin and chymotrypsin or combinations of both, suffered no loss in activity following treatment with these enzymes. Pepsin could not be tested because

[1] The elementary chemical analyses were made by Dr. A. Elek of The Rockefeller Institute.

[2] The authors are indebted to Dr. John H. Northrop and Dr. M. Kunitz of The Rockefeller Institute for Medical Research, Princeton, N. J., for the samples of crystalline trypsin, chymotrypsin, and ribonuclease used in this work.

extracts are rapidly inactivated at the low pH required for its use. Prolonged treatment with crystalline ribonuclease under optimal conditions caused no demonstrable decrease in transforming activity. The fact that trypsin, chymotrypsin, and ribonuclease had no effect on the transforming principle is further evidence that this substance is not ribonucleic acid or a protein susceptible to the action of tryptic enzymes.

In addition to the crystalline enzymes, sera and preparations of enzymes obtained from the organs of various animals were tested to determine their effect on transforming activity. Certain of these were found to be capable of completely destroying biological activity. The various enzyme preparations tested included highly active phosphatases obtained from rabbit bone by the method of Martland and Robison (15) and from swine kidney as described by

TABLE II

*The Inactivation of Transforming Principle by Crude Enzyme Preparations*

| Crude enzyme preparations | Enzymatic activity | | | |
|---|---|---|---|---|
| | Phosphatase | Tributyrin esterase | Depolymerase for desoxyribonucleate | Inactivation of transforming principle |
| Dog intestinal mucosa | + | + | + | + |
| Rabbit bone phosphatase | + | + | − | − |
| Swine kidney " | + | − | − | − |
| Pneumococcus autolysates | − | + | + | + |
| Normal dog and rabbit serum | + | + | + | + |

H. and E. Albers (16). In addition, a preparation made from the intestinal mucosa of dogs by Levene and Dillon (17) and containing a polynucleotidase for thymus nucleic acid was used. Pneumococcal autolysates and a commercial preparation of pancreatin were also tested. The alkaline phosphatase activity of these preparations was determined by their action on β-glycerophosphate and phenyl phosphate, and the esterase activity by their capacity to split tributyrin. Since the highly purified transforming material isolated from pneumococcal extracts was found to contain desoxyribonucleic acid, these same enzymes were tested for depolymerase activity on known samples of desoxyribonucleic acid isolated by Mirsky[3] from fish sperm and mammalian tissues. The results are summarized in Table II in which the phosphatase, esterase, and nucleodepolymerase activity of these enzymes is compared with their capacity to destroy the transforming principle. Analysis of these results shows that irrespective of the presence of phosphatase or esterase only those

[3] The authors express their thanks to Dr. A. E. Mirsky of the Hospital of The Rockefeller Institute for these preparations of desoxyribonucleic acid.

preparations shown to contain an enzyme capable of depolymerizing authentic samples of desoxyribonucleic acid were found to inactivate the transforming principle.

Greenstein and Jenrette (18) have shown that tissue extracts, as well as the milk and serum of several mammalian species, contain an enzyme system which causes depolymerization of desoxyribonucleic acid. To this enzyme system Greenstein has later given the name desoxyribonucleodepolymerase (19). These investigators determined depolymerase activity by following the reduction in viscosity of solutions of sodium desoxyribonucleate. The nucleate and enzyme were mixed in the viscosimeter and viscosity measurements made at intervals during incubation at 30°C. In the present study this method was used in the measurement of depolymerase activity except that incubation was carried out at 37°C. and, in addition to the reduction of viscosity, the action of the enzyme was further tested by the progressive decrease in acid precipitability of the nucleate during enzymatic breakdown.

The effect of fresh normal dog and rabbit serum on the activity of the transforming substance is shown in the following experiment.

Sera obtained from a normal dog and normal rabbit were diluted with an equal volume of physiological saline. The diluted serum was divided into three equal portions. One part was heated at 65°C. for 30 minutes, another at 60°C. for 30 minutes, and the third was used unheated as control. A partially purified preparation of transforming material which had previously been dried in the lyophile apparatus was dissolved in saline in a concentration of 3.7 mg. per cc. 1.0 cc. of this solution was mixed with 0.5 cc. of the various samples of heated and unheated diluted sera, and the mixtures at pH 7.4 were incubated at 37°C. for 2 hours. After the serum had been allowed to act on the transforming material for this period, all tubes were heated at 65°C. for 30 minutes to stop enzymatic action. Serial dilutions were then made in saline and tested in triplicate for transforming activity according to the procedure described under Method of titration. The results given in Table III illustrate the differential heat inactivation of the enzymes in dog and rabbit serum which destroy the transforming principle.

From the data presented in Table III it is evident that both dog and rabbit serum in the unheated state are capable of completely destroying transforming activity. On the other hand, when samples of dog serum which have been heated either at 60°C. or at 65°C. for 30 minutes are used, there is no loss of transforming activity. Thus, in this species the serum enzyme responsible for destruction of the transforming principle is completely inactivated at 60°C. In contrast to these results, exposure to 65°C. for 30 minutes was required for complete destruction of the corresponding enzyme in rabbit serum.

The same samples of dog and rabbit serum used in the preceding experiment were also tested for their depolymerase activity on a preparation of sodium desoxyribonucleate isolated by Mirsky from shad sperm.

A highly viscous solution of the nucleate in distilled water in a concentration of 1 mg. per cc. was used. 1.0 cc. amounts of heated and unheated sera diluted in saline as shown in the preceding protocol were mixed in Ostwald viscosimeters with 4.0 cc.

TABLE III

*Differential Heat Inactivation of Enzymes in Dog and Rabbit Serum Which Destroy the Transforming Substance*

| | Heat treatment of serum | Dilution* | Triplicate tests | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | | 2 | | 3 | |
| | | | Diffuse growth | Colony form | Diffuse growth | Colony form | Diffuse growth | Colony form |
| Dog serum | Unheated | Undiluted | − | R only | − | R only | − | R only |
| | | 1:5 | − | R " | − | R " | − | R " |
| | | 1:25 | − | R " | − | R " | − | R " |
| | 60°C. for 30 min. | Undiluted | + | SIII | + | SIII | + | SIII |
| | | 1:5 | + | SIII | + | SIII | + | SIII |
| | | 1:25 | + | SIII | + | SIII | + | SIII |
| | 65°C. for 30 min. | Undiluted | + | SIII | + | SIII | + | SIII |
| | | 1:5 | + | SIII | + | SIII | + | SIII |
| | | 1:25 | + | SIII | + | SIII | + | SIII |
| Rabbit serum | Unheated | Undiluted | − | R only | − | R only | − | R only |
| | | 1:5 | − | R " | − | R " | − | R " |
| | | 1:25 | − | R " | − | R " | − | R " |
| | 60°C. for 30 min. | Undiluted | − | R only | − | R only | − | R only |
| | | 1:5 | − | R " | − | R " | − | R " |
| | | 1:25 | − | R " | − | R " | − | R " |
| | 65°C. for 30 min. | Undiluted | + | SIII | + | SIII | + | SIII |
| | | 1:5 | + | SIII | + | SIII | + | SIII |
| | | 1:25 | + | SIII | + | SIII | + | SIII |
| Control (no serum) | None | Undiluted | + | SIII | + | SIII | + | SIII |
| | | 1:5 | + | SIII | + | SIII | + | SIII |
| | | 1:25 | + | SIII | + | SIII | + | SIII |

* Dilution of the digest mixture of serum and transforming substance.

of the aqueous solution of the nucleate. Determinations of viscosity were made immediately and at intervals over a period of 24 hours during incubation at 37°C.

The results of this experiment are graphically presented in Chart 1. In the case of unheated serum of both dog and rabbit, the viscosity fell to that of water in 5 to 7 hours. Dog serum heated at 60°C. for 30 minutes brought about

no significant reduction in viscosity after 22 hours. On the other hand, heating rabbit serum at 60°C. merely reduced the rate of depolymerase action, and after 24 hours the viscosity was brought to the same level as with the unheated serum. Heating at 65°C., however, completely destoyed the rabbit serum depolymerase.

Thus, in the case of dog and rabbit sera there is a striking parallelism between the temperature of inactivation of the depolymerase and that of the enzyme which destroys the activity of the transforming principle. The fact that this difference in temperature of inactivation is not merely a general property of all enzymes in the sera is evident from experiments on the heat inactivation of

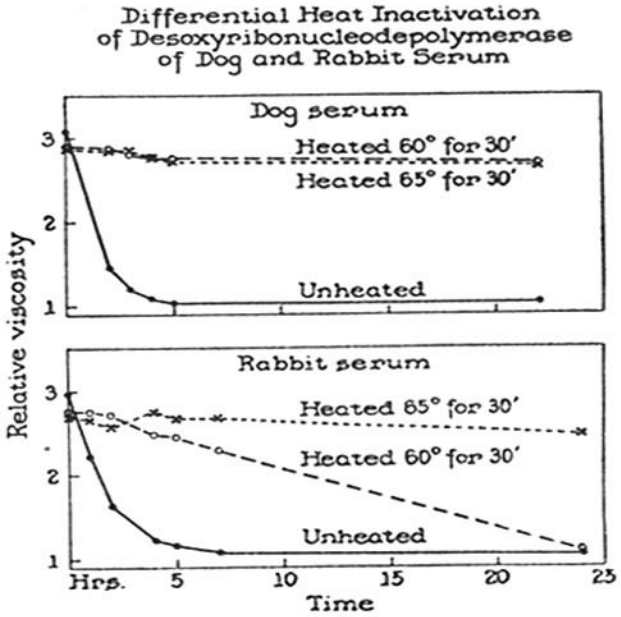



CHART 1

tributyrin esterase in the same samples of serum. In the latter instance, the results are the reverse of those observed with depolymerase since the esterase of rabbit serum is almost completely inactivated at 60°C. while that in dog serum is only slightly affected by exposure to this temperature.

Of a number of substances tested for their capacity to inhibit the action of the enzyme known to destroy the transforming principle, only sodium fluoride has been found to have a significant inhibitory effect. Regardless of whether this enzyme is derived from pneumococcal cells, dog intestinal mucosa, pancreatin, or normal sera its activity is inhibited by fluoride. Similarly it has been found that fluoride in the same concentration also inhibits the enzymatic depolymerization of desoxyribonucleic acid.

The fact that transforming activity is destroyed only by those preparations containing depolymerase for desoxyribonucleic acid and the further fact that

in both instances the enzymes concerned are inactivated at the same temperature and inhibited by fluoride provide additional evidence for the belief that the active principle is a nucleic acid of the desoxyribose type.

*Serological Analysis.*—In the course of chemical isolation of the active material it was found that as crude extracts were purified, their serological activity in Type III antiserum progressively decreased without corresponding loss in biological activity. Solutions of the highly purified substance itself gave only faint trace reactions in precipitin tests with high titer Type III antipneumococcus rabbit serum.[4] It is well known that pneumococcal protein can be detected by serological methods in dilutions as high as 1:50,000 and the capsular as well as the somatic polysaccharide in dilutions of at least 1:5,000,000. In view of these facts, the loss of serological reactivity indicates that these cell constituents have been almost completely removed from the final preparations. The fact that the transforming substance in purified state exhibits little or no serological reactivity is in striking contrast to its biological specificity in inducing pneumococcal transformation.

*Physicochemical Studies.*[5]—A purified and active preparation of the transforming substance (preparation 44) was examined in the analytical ultracentrigue. The material gave a single and unusually sharp boundary indicating that the substance was homogeneous and that the molecules were uniform in size and very asymmetric. Biological activity was found to be sedimented at the same rate as the optically observed boundary, showing that activity could not be due to the presence of an entity much different in size. The molecular weight cannot be accurately determined until measurements of the diffusion constant and partial specific volume have been made. However, Tennent and Vilbrandt (20) have determined the diffusion constant of several preparations of thymus nucleic acid the sedimentation rate of which is in close agreement with the values observed in the present study. Assuming that the asymmetry of the molecules is the same in both instances, it is estimated that the molecular weight of the pneumococcal preparation is of the order of 500,000.

Examination of the same active preparation was carried out by electrophoresis in the Tiselius apparatus and revealed only a single electrophoretic component of relatively high mobility comparable to that of a nucleic acid. Transforming activity was associated with the fast moving component giving the

[4] The Type III antipneumococcus rabbit serum employed in this study was furnished through the courtesy of Dr. Jules T. Freund, Bureau of Laboratories, Department of Health, City of New York.

[5] Studies on sedimentation in the ultracentrifuge were carried out by Dr. A. Rothen; the electrophoretic analyses were made by Dr. T. Shedlovsky, and the ultraviolet absorption curves by Dr. G. I. Lavin. The authors gratefully acknowledge their indebtedness to these members of the staff of The Rockefeller Institute.

optically visible boundary. Thus in both the electrical and centrifugal fields, the behavior of the purified substance is consistent with the concept that biological activity is a property of the highly polymerized nucleic acid.

Ultraviolet absorption curves showed maxima in the region of 2600 Å and minima in the region of 2350 Å. These findings are characteristic of nucleic acids.

*Quantitative Determination of Biological Activity.*—In its highly purified state the material as isolated has been found to be capable of inducing transformation in amounts ranging from 0.02 to 0.003 μg. Preparation 44, the purification of which was carried out at low temperature and which had a nitrogen-phosphorus

TABLE IV

*Titration of Transforming Activity of Preparation 44*

| Transforming principle Preparation 44* | | Quadruplicate tests | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 3 | | 4 | |
| Dilution | Amount added | Diffuse growth | Colony form | Diffuse growth | Colony form | Diffuse growth | Colony form | Diffuse growth | Colony form |
| | μg. | | | | | | | | |
| $10^{-2}$ | 1.0 | + | SIII | + | SIII | + | SIII | + | SIII |
| $10^{-2.5}$ | 0.3 | + | SIII | + | SIII | + | SIII | + | SIII |
| $10^{-3}$ | 0.1 | + | SIII | + | SIII | + | SIII | + | SIII |
| $10^{-3.5}$ | 0.03 | + | SIII | + | SIII | + | SIII | + | SIII |
| $10^{-4}$ | 0.01 | + | SIII | + | SIII | + | SIII | + | SIII |
| $10^{-4.5}$ | 0.003 | — | R only | + | SIII | — | R only | + | SIII |
| $10^{-5}$ | 0.001 | — | R " | — | R only | — | R " | — | R only |
| Control | None | — | R " | — | R " | — | R " | — | R " |

* Solution from which dilutions were made contained 0.5 mg. per cc. of purified material. 0.2 cc. of each dilution added to quadruplicate tubes containing 2.0 cc. of standard serum broth. 0.05 cc. of a $10^{-4}$ dilution of a blood broth culture of R36A is added to each tube.

ratio of 1.58, exhibited high transforming activity. Titration of the activity of this preparation is given in Table IV.

A solution containing 0.5 mg. per cc. was serially diluted as shown in the protocol. 0.2 cc. of each of these dilutions was added to quadruplicate tubes containing 2.0 cc. of standard serum broth. All tubes were then inoculated with 0.05 cc. of a $10^{-4}$ dilution of a 5 to 8 hour blood broth culture of R36A. Transforming activity was determined by the procedure described under Method of titration.

The data presented in Table IV show that on the basis of dry weight 0.003 μg. of the active material brought about transformation. Since the reaction system containing the 0.003 μg. has a volume of 2.25 cc., this represents a final concentration of the purified substance of 1 part in 600,000,000.

## DISCUSSION

The present study deals with the results of an attempt to determine the chemical nature of the substance inducing specific transformation of pneumococcal types. A desoxyribonucleic acid fraction has been isolated from Type III pneumococci which is capable of transforming unencapsulated R variants derived from Pneumococcus Type II into fully encapsulated Type III cells. Thompson and Dubos (21) have isolated from pneumococci a nucleic acid of the ribose type. So far as the writers are aware, however, a nucleic acid of the desoxyribose type has not heretofore been recovered from pneumococci nor has specific transformation been experimentally induced *in vitro* by a chemically defined substance.

Although the observations are limited to a single example, they acquire broader significance from the work of earlier investigators who demonstrated the interconvertibility of various pneumococcal types and showed that the specificity of the changes induced is in each instance determined by the particular type of encapsulated cells used to evoke the reaction. From the point of view of the phenomenon in general, therefore, it is of special interest that in the example studied, highly purified and protein-free material consisting largely, if not exclusively, of desoxyribonucleic acid is capable of stimulating unencapsulated R variants of Pneumococcus Type II to produce a capsular polysaccharide identical in type specificity with that of the cells from which the inducing substance was isolated. Equally striking is the fact that the substance evoking the reaction and the capsular substance produced in response to it are chemically distinct, each belonging to a wholly different class of chemical compounds.

The inducing substance, on the basis of its chemical and physical properties, appears to be a highly polymerized and viscous form of sodium desoxyribonucleate. On the other hand, the Type III capsular substance, the synthesis of which is evoked by this transforming agent, consists chiefly of a non-nitrogenous polysaccharide constituted of glucose-glucuronic acid units linked in glycosidic union (22). The presence of the newly formed capsule containing this type-specific polysaccharide confers on the transformed cells all the distinguishing characteristics of Pneumococcus Type III. Thus, it is evident that the inducing substance and the substance produced in turn are chemically distinct and biologically specific in their action and that both are requisite in determining the type specificity of the cell of which they form a part.

The experimental data presented in this paper strongly suggest that nucleic acids, at least those of the desoxyribose type, possess different specificities as evidenced by the selective action of the transforming principle. Indeed, the possibility of the existence of specific differences in biological behavior of nucleic acids has previously been suggested (23, 24) but has never been experimentally demonstrated owing in part at least to the lack of suitable biological methods.

The techniques used in the study of transformation appear to afford a sensitive means of testing the validity of this hypothesis, and the results thus far obtained add supporting evidence in favor of this point of view.

If it is ultimately proved beyond reasonable doubt that the transforming activity of the material described is actually an inherent property of the nucleic acid, one must still account on a chemical basis for the biological specificity of its action. At first glance, immunological methods would appear to offer the ideal means of determining the differential specificity of this group of biologically important substances. Although the constituent units and general pattern of the nucleic acid molecule have been defined, there is as yet relatively little known of the possible effect that subtle differences in molecular configuration may exert on the biological specificity of these substances. However, since nucleic acids free or combined with histones or protamines are not known to function antigenically, one would not anticipate that such differences would be revealed by immunological techniques. Consequently, it is perhaps not surprising that highly purified and protein-free preparations of desoxyribonucleic acid, although extremely active in inducing transformation, showed only faint trace reactions in precipitin tests with potent Type III antipneumococcus rabbit sera.

From these limited observations it would be unwise to draw any conclusion concerning the immunological significance of the nucleic acids until further knowledge on this phase of the problem is available. Recent observations by Lackman and his collaborators (25) have shown that nucleic acids of both the yeast and thymus type derived from hemolytic streptococci and from animal and plant sources precipitate with certain antipneumococcal sera. The reactions varied with different lots of immune serum and occurred more frequently in antipneumococcal horse serum than in corresponding sera of immune rabbits. The irregularity and broad cross reactions encountered led these investigators to express some doubt as to the immunological significance of the results. Unless special immunochemical methods can be devised similar to those so successfully used in demonstrating the serological specificity of simple non-antigenic substances, it appears that the techniques employed in the study of transformation are the only ones available at present for testing possible differences in the biological behavior of nucleic acids.

Admittedly there are many phases of the problem of transformation that require further study and many questions that remain unanswered largely because of technical difficulties. For example, it would be of interest to know the relation between rate of reaction and concentration of the transforming substance; the proportion of cells transformed to those that remain unaffected in the reaction system. However, from a bacteriological point of view, numerical estimations based on colony counts might prove more misleading than enlightening because of the aggregation and sedimentation of the R cells ag-

glutinated by the antiserum in the medium. Attempts to induce transformation in suspensions of resting cells held under conditions inhibiting growth and multiplication have thus far proved unsuccessful, and it seems probable that transformation occurs only during active reproduction of the cells. Important in this connection is the fact that the R cells, as well as those that have undergone transformation, presumably also all other variants and types of pneumococci, contain an intracellular enzyme which is released during autolysis and in the free state is capable of rapidly and completely destroying the activity of the transforming agent. It would appear, therefore, that during the logarithmic phase of growth when cell division is most active and autolysis least apparent, the cultural conditions are optimal for the maintenance of the balance between maximal reactivity of the R cell and minimal destruction of the transforming agent through the release of autolytic ferments.

In the present state of knowledge any interpretation of the mechanism involved in transformation must of necessity be purely theoretical. The biochemical events underlying the phenomenon suggest that the transforming principle interacts with the R cell giving rise to a coordinated series of enzymatic reactions that culminate in the synthesis of the Type III capsular antigen. The experimental findings have clearly demonstrated that the induced alterations are not random changes but are predictable, always corresponding in type specificity to that of the encapsulated cells from which the transforming substance was isolated. Once transformation has occurred, the newly acquired characteristics are thereafter transmitted in series through innumerable transfers in artificial media without any further addition of the transforming agent. Moreover, from the transformed cells themselves, a substance of identical activity can again be recovered in amounts far in excess of that originally added to induce the change. It is evident, therefore, that not only is the capsular material reproduced in successive generations but that the primary factor, which controls the occurrence and specificity of capsular development, is also reduplicated in the daughter cells. The induced changes are not temporary modifications but are permanent alterations which persist provided the cultural conditions are favorable for the maintenance of capsule formation. The transformed cells can be readily distinguished from the parent R forms not alone by serological reactions but by the presence of a newly formed and visible capsule which is the immunological unit of type specificity and the accessory structure essential in determining the infective capacity of the microorganism in the animal body.

It is particularly significant in the case of pneumococci that the experimentally induced alterations are definitely correlated with the development of a new morphological structure and the consequent acquisition of new antigenic and invasive properties. Equally if not more significant is the fact that these changes are predictable, type-specific, and heritable.

Various hypotheses have been advanced in explanation of the nature of the changes induced. In his original description of the phenomenon Griffith (1) suggested that the dead bacteria in the inoculum might furnish some specific protein that serves as a "pabulum" and enables the R form to manufacture a capsular carbohydrate.

More recently the phenomenon has been interpreted from a genetic point of view (26, 27). The inducing substance has been likened to a gene, and the capsular antigen which is produced in response to it has been regarded as a gene product. In discussing the phenomenon of transformation Dobzhansky (27) has stated that "If this transformation is described as a genetic mutation—and it is difficult to avoid so describing it—we are dealing with authentic cases of induction of specific mutations by specific treatments. . . ."

Another interpretation of the phenomenon has been suggested by Stanley (28) who has drawn the analogy between the activity of the transforming agent and that of a virus. On the other hand, Murphy (29) has compared the causative agents of fowl tumors with the transforming principle of Pneumococcus. He has suggested that both these groups of agents be termed "transmissible mutagens" in order to differentiate them from the virus group. Whatever may prove to be the correct interpretation, these differences in viewpoint indicate the implications of the phenomenon of transformation in relation to similar problems in the fields of genetics, virology, and cancer research.

It is, of course, possible that the biological activity of the substance described is not an inherent property of the nucleic acid but is due to minute amounts of some other substance adsorbed to it or so intimately associated with it as to escape detection. If, however, the biologically active substance isolated in highly purified form as the sodium salt of desoxyribonucleic acid actually proves to be the transforming principle, as the available evidence strongly suggests, then nucleic acids of this type must be regarded not merely as structurally important but as functionally active in determining the biochemical activities and specific characteristics of pneumococcal cells. Assuming that the sodium desoxyribonucleate and the active principle are one and the same substance, then the transformation described represents a change that is chemically induced and specifically directed by a known chemical compound. If the results of the present study on the chemical nature of the transforming principle are confirmed, then nucleic acids must be regarded as possessing biological specificity the chemical basis of which is as yet undetermined.

## SUMMARY

1. From Type III pneumococci a biologically active fraction has been isolated in highly purified form which in exceedingly minute amounts is capable under appropriate cultural conditions of inducing the transformation of unencapsulated R variants of Pneumococcus Type II into fully encapsulated cells of the

same specific type as that of the heat-killed microorganisms from which the inducing material was recovered.

2. Methods for the isolation and purification of the active transforming material are described.

3. The data obtained by chemical, enzymatic, and serological analyses together with the results of preliminary studies by electrophoresis, ultracentrifugation, and ultraviolet spectroscopy indicate that, within the limits of the methods, the active fraction contains no demonstrable protein, unbound lipid, or serologically reactive polysaccharide and consists principally, if not solely, of a highly polymerized, viscous form of desoxyribonucleic acid.

4. Evidence is presented that the chemically induced alterations in cellular structure and function are predictable, type-specific, and transmissible in series. The various hypotheses that have been advanced concerning the nature of these changes are reviewed.

## CONCLUSION

The evidence presented supports the belief that a nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type III.

## BIBLIOGRAPHY

1. Griffith, F., *J. Hyg.*, Cambridge, Eng., 1928, **27,** 113.
2. Neufeld, F., and Levinthal, W., *Z. Immunitätsforsch.*, 1928, **55,** 324.
3. Baurhenn, W., *Centr. Bakt., 1. Abt., Orig.*, 1932, **126,** 68.
4. Dawson, M. H., *J. Exp. Med.*, 1930, **51,** 123.
5. Dawson, M. H., and Sia, R. H. P., *J. Exp. Med.*, 1931, **54,** 681.
6. Alloway, J. L., *J. Exp. Med.*, 1932, **55,** 91; 1933, **57,** 265.
7. Berry, G. P., and Dedrick, H. M., *J. Bact.*, 1936, **31,** 50.
8. Berry, G. P., *Arch. Path.*, 1937, **24,** 533.
9. Hurst, E. W., *Brit. J. Exp. Path.*, 1937, **18,** 23. Hoffstadt, R. E., and Pilcher, K. S., *J. Infect. Dis.*, 1941, **68,** 67. Gardner, R. E., and Hyde, R. R., *J. Infect. Dis.*, 1942, **71,** 47. Houlihan, R. B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51,** 259.
10. MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44,** 277.
11. Dawson, M. H., *J. Exp. Med.*, 1928, **47,** 577; 1930, **51,** 99.
12. Sevag, M. G., *Biochem. Z.*, 1934, **273,** 419. Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.*, 1938, **124,** 425.
13. Dubos, R. J., and Avery, O. T., *J. Exp. Med.*, 1931, **54,** 51. Dubos, R. J., and Bauer, J. H., *J. Exp. Med.*, 1935, **62,** 271.
14. Liu, S., and Wu, H., *Chinese J. Physiol.*, 1938, **13,** 449.
15. Martland, M., and Robison, R., *Biochem. J.*, 1929, **23,** 237.
16. Albers, H., and Albers, E., *Z. physiol. Chem.*, 1935, **232,** 189.
17. Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, 1933, **96,** 461.
18. Greenstein, J. P., and Jenrette, W. Y., *J. Nat. Cancer Inst.*, 1940, **1,** 845.

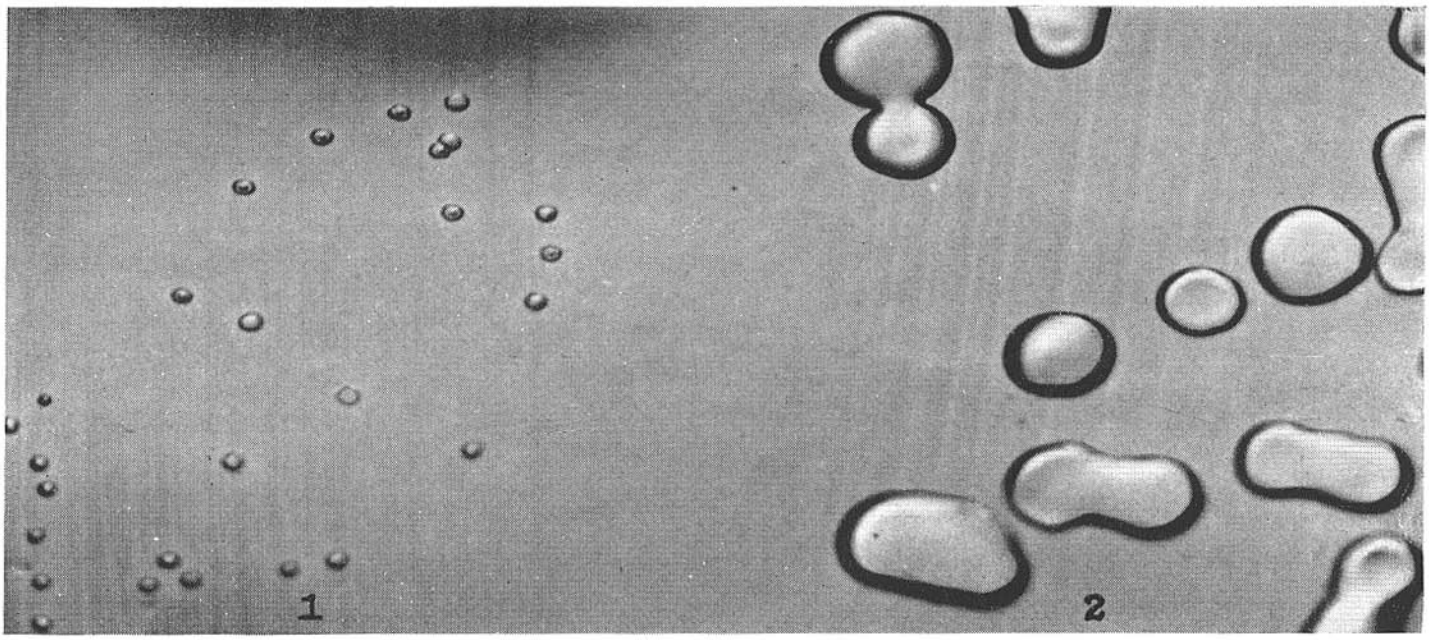
19. Greenstein, J. P., *J. Nat. Cancer Inst.*, 1943, **4,** 55.
20. Tennent, H. G., and Vilbrandt, C. F., *J. Am. Chem. Soc.*, 1943, **65,** 424.
21. Thompson, R. H. S., and Dubos, R. J., *J. Biol. Chem.*, 1938, **125,** 65.
22. Reeves, R. E., and Goebel, W. F., *J. Biol. Chem.*, 1941, **139,** 511.
23. Schultz, J., in Genes and chromosomes. Structure and organization, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1941, **9,** 55.
24. Mirsky, A. E., in Advances in enzymology and related subjects of biochemistry, (F. F. Nord and C. H. Werkman, editors), New York, Interscience Publishers, Inc., 1943, **3,** 1.
25. Lackman, D., Mudd, S., Sevag, M. G., Smolens, J., and Wiener, M., *J. Immunol.*, 1941, **40,** 1.
26. Gortner, R. A., Outlines of biochemistry, New York, Wiley, 2nd edition, 1938, 547.
27. Dobzhansky, T., Genetics and the origin of the species, New York, Columbia University Press, 1941, 47.
28. Stanley, W. M., in Doerr, R., and Hallauer, C., Handbuch der Virusforschung, Vienna, Julius Springer, 1938, **1,** 491.
29. Murphy, J. B., *Tr. Assn. Am. Physn.*, 1931, **46,** 182; *Bull. Johns Hopkins Hosp.*, 1935, **56,** 1.

## EXPLANATION OF PLATE 1

The photograph was made by Mr. Joseph B. Haulenbeek.

FIG. 1. Colonies of the R variant (R36A) derived from Pneumococcus Type II. Plated on blood agar from a culture grown in serum broth in the absence of the transforming substance. ×3.5.

FIG. 2. Colonies on blood agar of the same cells after induction of transformation during growth in the same medium with the addition of active transforming principle isolated from Type III pneumococci. The smooth, glistening, mucoid colonies shown are characteristic of Pneumococcus Type III and readily distinguishable from the small, rough colonies of the parent R strain illustrated in Fig. 1. ×3.5.




(Avery *et al.*: Transformation of pneumococcal types)